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**Research Article: New Research | Neuronal Excitability**

**Arc controls AMPAR endocytosis through a direct interaction with clathrin-adaptor protein 2**

The Arc/AP-2 interaction mediates synaptic transmission

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18 clamp experiments and wrote the manuscript. LLPdS conceived and designed the  
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20 performed experiments, analyzed and interpreted the data and helped to write the  
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22 experiments. SCW performed the immunoprecipitation experiments. MJW performed  
23 and analyzed the electrophysiological experiments. YCJ: performed experiments,  
24 analyzed and interpreted the data. JM designed the strategy to knockdown AP-2,  
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26 performed the modelling analysis of the Arc structure and contributed to the writing  
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53

#### 54 **Abstract**

55 The activity-regulated cytoskeleton-associated (Arc) protein control synaptic strength by  
56 facilitating AMPA receptor (AMPA) endocytosis. Here we demonstrate that Arc targets  
57 AMPAR to be internalized through a direct interaction with the clathrin-adaptor protein 2 (AP-  
58 2). We show that Arc overexpression overexpression in dissociated hippocampal neurons  
59 obtained from C57BL/6 mouse reduces the density of AMPAR GluA1 subunits at the cell  
60 surface and reduces the amplitude and rectification of AMPAR-mediated miniature-excitatory  
61 postsynaptic currents (mEPSC). Mutations of Arc, that prevent the AP-2 interaction reduce  
62 Arc-mediated endocytosis of GluA1 and abolish the reduction in AMPAR-mediated mEPSC  
63 amplitude and rectification. Depletion of the AP-2 subunit  $\mu 2$  blocks the Arc-mediated  
64 reduction in mEPSC amplitude, effect that is restored by re-introducing  $\mu 2$ . The Arc/AP-2  
65 interaction plays an important role in homeostatic synaptic scaling as the Arc-dependent  
66 decrease in mEPSC amplitude, induced by a chronic increase in neuronal activity, is  
67 inhibited by AP-2 depletion. This data provides a mechanism to explain how activity-  
68 dependent expression of Arc decisively controls the fate of AMPAR at the cell surface and  
69 modulates synaptic strength, via the direct interaction with the endocytic clathrin adaptor AP-  
70 2.

71

#### 72 **Significance Statement**

73 The direct binding of Arc to the clathrin-adaptor protein 2 complex discovered in this study  
74 provides the crucial mechanistic link between the activity-dependent expression of Arc and  
75 the targeting of specific synaptic AMPA receptors for endocytosis. The interaction between  
76 Arc and AP-2 is crucial for many forms of synaptic plasticity and may provide a novel target  
77 for therapeutic intervention.

78

79 **Introduction**

80 Activity-dependent long-lasting alterations in glutamatergic synaptic strength are the  
81 molecular substrate thought to underlie learning and memory. The establishment and  
82 maintenance of changes in synaptic strength is dependent on trafficking of AMPAR at the  
83 postsynaptic membrane (Ehlers, 2000; Newpher and Ehlers, 2008), together with changes in  
84 protein synthesis (Buffington et al., 2014). In recent years, several neuron specific immediate  
85 early genes (IEGs) that are rapidly induced in response to neuronal activity have been  
86 described (Flavell and Greenberg, 2008), including Arc, also named activity-regulated gene  
87 of 3.1 kb (Arg3.1). Following neuronal activation, Arc mRNA is rapidly trafficked to  
88 postsynaptic dendritic sites and locally translated (Lyford et al., 1995; Steward et al., 1998).  
89 A rapid increase in Arc protein expression regulates synaptic strength, mainly by enhancing  
90 the endocytosis of AMPAR at postsynaptic sites (Rial Verde et al., 2006; Shepherd et al.,  
91 2006; Waung et al., 2008; Mabb et al., 2014). A number of studies have shown that Arc  
92 regulates several forms of synaptic plasticity, including homeostatic scaling (Shepherd et al.,  
93 2006; Corrêa et al., 2012; Mabb et al., 2014) and metabotropic glutamate receptor-  
94 dependent long-term depression (mGluR-LTD) (Waung et al., 2008; Jakkamsetti et al., 2013;  
95 Mabb et al., 2014). Arc is also required for inverse synaptic tagging. In this process, strong  
96 neuronal stimulation induces Arc expression which binds to inactive CaMKII $\beta$  (Okuno et al.,  
97 2012). The Arc/CaMKII $\beta$  complex then operates as a sensor to identify and induce  
98 endocytosis of AMPAR at weaker synapses thus increasing the difference between activated  
99 and non-activated synapses. Together, these findings demonstrate a pivotal role for Arc in  
100 regulating synapse strength after neuronal activation.

101

102 The clathrin-mediated endocytic (CME) pathway has been the subject of intensive studies in  
103 the past decades. Therefore, the molecular machinery involved in the sequential events  
104 linking the selection of the endocytic cargo and assembly of the clathrin scaffold leading to  
105 membrane bending and scission of the newly formed clathrin-coated vesicles has been

precisely described (Saheki and De Camilli, 2012; Canagarajah et al., 2013; Kirchhausen et al., 2014). The clathrin-adaptor protein 2 (AP-2), which is a heterotetramer composed of two large ( $\alpha/\beta 2$ ) and two small ( $\mu 2/\sigma 2$ ) subunits, plays an essential role in the formation of endocytic clathrin-coated vesicles (CCV). To initiate the clathrin-coat assembly the AP-2 complex first binds to the transmembrane cargo that is to be internalized and subsequently binds and connects clathrin to the plasma membrane (Saheki and De Camilli, 2012; Traub and Bonifacino, 2013; Kirchhausen et al., 2014). The sequential events observed during clathrin-mediated endocytosis are conserved across different eukaryotic cell types including neurons (Saheki and De Camilli, 2012). In hippocampal neurons, the cytosolic tail of the AMPAR subunit 2 (GluA2) directly binds to AP-2 (Kastning et al., 2007) and disruption of the AMPAR/AP-2 interaction compromises the Arc-mediated facilitation of AMPAR endocytosis (Rial Verde et al., 2006).

118

Here we show that Arc directly binds to AP-2 and that this interaction is required for Arc-mediated endocytosis of GluA1 subunits and consequent changes in synaptic transmission. Under basal conditions, overexpression of Arc-wild-type (Arc-WT) reduces the amplitude and rectification of AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs), whereas Arc proteins bearing mutations in the AP-2 binding site, have little or no effect. Furthermore, depletion of AP-2 blocks the Arc-mediated reduction in mEPSC amplitude, an effect that is rescued when AP-2 expression is restored. The interaction between Arc and AP-2 is also important in homeostatic synaptic scaling, as depletion of AP-2 significantly reduces the Arc-dependent decrease in AMPAR mEPSC amplitude induced by increased neuronal activity. The discovery that the direct interaction between Arc and AP-2 facilitates rapid and sustained AMPAR endocytosis provides the mechanistic link by which constitutive endocytosis can be regulated by changes in activity in neurons. These findings further consolidate the strategic role of Arc in facilitating activity-dependent endocytosis of AMPAR in synaptic plasticity.

133

## **Materials and Methods**

Animals used in this study were treated in accordance with UK Animal (Scientific Procedures) Act 1986 legislation and under the appropriate national and local ethical approval. Sample size was calculated using variance from previous experiments to indicate power, with statistical significance set at 95%. Replication values are incorporated in the figures, where appropriate.

### **Immunoprecipitation and immunoblot analysis**

To identify new proteins that interact with endogenous Arc/Arg3.1 proteins hippocampi from 10 week-old male C57BL/6 mice were used. To extract the hippocampi, animals were deeply anaesthetized and the brains were rapidly removed and placed in ice-cold artificial CSF (aCSF) consisting of (mM): 124 NaCl, 3 KCl, 26 NaHCO<sub>3</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 2 CaCl<sub>2</sub>, 1 MgSO<sub>4</sub> and 10 D-glucose (bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>). Hippocampi were then isolated from the surrounding tissue and cut into small pieces using a dissecting microscope (Leica LED 1000). The tissue was then homogenized in Eppendorf Scientific tubes with a pellet pestle in ice-cold solution composed of: 10 mM HEPES, 0.32 M sucrose and protease inhibitor cocktail (Roche) and rotated for 1 h at 4°C. Homogenate was centrifuged at 13,000 g for 15 min, the supernatant collected and protein levels determined (BCA protein assay kit, Thermo Scientific). 500 µg of protein making 500 µl of final volume was incubated with 1 µg of rabbit polyclonal anti-Arc antibody (Synaptic Systems, 156-003) and 15 µl of pre-washed protein G agarose beads (Upstate-Millipore, 16-266) and rotated for 3 h at 4°C. As a negative control, 500 µg of protein was incubated with 15 µl with protein G agarose beads only. Arc-IP and negative control samples were centrifuged at 7000 g for 30 sec to precipitate the beads. The supernatant was removed and the beads washed 3 times with lysis buffer containing 1 mM EDTA, 1 M Tris-HCl (pH 7.5), 1% Triton X-100, 1 mM Sodium Orthovanadate, 50 mM Sodium Fluoride, Sodium pyrophosphate, 0.27 M Sucrose, 20% NaN<sub>3</sub> and protease inhibitor cocktail (Roche). Proteins were eluted from the beads with 20 µl of 5X loading buffer, and



161 the total amount of the eluted protein from the beads were loaded into a 10% SDS-PAGE  
162 gels and separated for 1.5 cm using electrophoresis system.

163 To further confirm the endogenous interaction between Arc and AP-2 in the hippocampus,  
164 we used the co-IPs experimental conditions described above. Eluted IP proteins as well as  
165 inputs were separated in 10% SDS-PAGE gels, transferred into membrane using  
166 electrophoresis system and blots were incubated overnight with primary antibodies: rabbit  
167 anti-Arc/Arg3.1 (1:1000 dilution), mouse anti- $\alpha$ -adaptin1/2 (1:1000 dilution, sc-17771) and  
168 goat anti-clathrin HC (1:1000 dilution, sc-6579). Normal Rabbit IgG (1:1000; RD Systems,  
169 AB-105-C) was used as negative control for the IP experiments. Appropriate secondary  
170 antibodies were used to detect proteins levels.

171

#### 172 Proteomics and MS analysis

173 Each gel lane (Arc IP and control) were cut in small pieces and subjected to in-gel tryptic  
174 digestion using a ProGest automated digestion unit (Digilab UK). The resulting peptides  
175 were fractionated using a Dionex Ultimate 3000 nanoHPLC system. Briefly, peptides in 1%  
176 (v/v) formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Dionex).  
177 After washing with 0.5% (v/v) acetonitrile 0.1% (v/v) formic acid peptides were resolved on a  
178 250 mm  $\times$  75  $\mu$ m Acclaim PepMap C18 reverse phase analytical column (Dionex) over a  
179 120 min organic gradient with a flow rate of 300 nl min<sup>-1</sup>. Peptides were ionized by nano-  
180 electrospray ionization at 2.3 kV using a stainless steel emitter with an internal diameter of  
181 30  $\mu$ m (Proxeon). Tandem mass spectrometry analysis was carried out on a LTQ-Orbitrap  
182 Velos mass spectrometer (Thermo Scientific). The Orbitrap was set to analyze the survey  
183 scans at 60,000 resolution and the top twenty ions in each duty cycle selected for MS/MS in  
184 the LTQ linear ion trap. Data was acquired using the Xcalibur v2.1 software (Thermo  
185 Scientific). The raw data files were processed and quantified using Proteome Discoverer  
186 software v1.2 (Thermo Scientific) with searches performed against the UniProt rat database  
187 by using the SEQUEST algorithm with the following criteria; peptide tolerance = 10 ppm,  
188 trypsin as the enzyme, carbamidomethylation of cysteine as a fixed modification and

189 oxidation of methionine as a variable modification. The reverse database search option was  
190 enabled and all data was filtered to satisfy false discovery rate (FDR) of less than 5%. Only  
191 hits from the Arc-co-IPs were considered for further characterization. The proteomics  
192 experiments were repeated twice

193

#### 194 Hippocampal cell culture and transfection

195 Hippocampal neuronal cultures were prepared from either male or female postnatal day 0  
196 pups from C57BL/6 wild-type mice as described previously (Canal et al., 2011). Briefly,  
197 hippocampi were extracted from the brain at 4°C, subject to digestion with trypsin (Sigma-  
198 Aldrich) and mechanically dissociated with DNase (Sigma-Aldrich). Cells were plated onto  
199 22-mm glass coverslips coated with poly-L-lysine hydrobromide (0.5mg/ml, Sigma-Aldrich).  
200 The plating medium consisted of Neurobasal-A medium (Gibco) supplemented with  
201 Gentamycin (ForMedium), L-Glutamine (ForMedium), 2% B27 (Gibco) and 5% horse serum  
202 (Gibco). The following day, the plating medium was changed for horse serum free feeding  
203 medium. Cultures were maintained at 37°C and 5% CO<sub>2</sub> in a humidified incubator. For  
204 immunocytochemistry and patch-clamp recordings, hippocampal cultured neurons were  
205 used at 14-16 *days in vitro* (DIV) and transfection were performed using Lipofectamine 2000  
206 (Life technologies). For the patch-clamp recordings cells expressing Arc cDNAs were used  
207 15-22 h after transfection and cells expressing shRNAs were transfected at 6-7 DIV and  
208 recorded at 14-16 DIV.

209

#### 210 Cell lineages culture and transfection

211 Human neuroglioma 4 (H4) cells obtained from the American Type Culture Collection  
212 (Manassas, VA) were cultured in Dulbecco's modified Eagle medium (DMEM; Life  
213 technologies), supplemented with 100 U of penicillin/ml, 0.1 mg of streptomycin/ml and 10%  
214 (vol/vol) fetal bovine serum (FBS) and transiently transfected using Lipofectamine 2000 (Life  
215 technologies). Neuroblastoma x Spinal Cord (NSC) hybrid mouse cell lines (Cashman et al.,  
216 1992) cultured in supplemented DMEM were transfect with nc shRNA,  $\mu$ 2-shRNA<sub>2</sub>,  $\mu$ 2-

217 shRNA<sub>3</sub> constructs using calcium phosphate as previously described (Canal et al., 2011).  
 218 After 72-96 h of transfection, cells were washed, lysed in the presence of protease inhibitor  
 219 cocktail (Roche) and 10 µg of protein were loaded onto a 10% acrylamide gel. Proteins were  
 220 separated using a SDS-PAGE electrophoresis system and transferred onto Hydrobond-ECL  
 221 membrane (GE healthcare). Membranes were incubated overnight with primary specific  
 222 mouse anti-AP-50 µ2 subunit antibody (1:500 dilution; BD 610350), and GAPDH (1:1000  
 223 dilution; Abcam ab8245 for Figure 6) or affinity purified rabbit polyclonal anti-GAPDH  
 224 antibody (1:1000 dilution; Sigma Aldrich G9545, for Figure 3). The membranes were  
 225 incubated with appropriate HRP-linked secondary antibodies anti-Mouse IgG (Cell Signaling  
 226 7076), anti-Mouse IgG (NA931V GE Healthcare) or anti-rabbit IgG (NA934V GE Healthcare)  
 227 incubated for 1 h at room temperature and blots developed using ECL reagents.

228

#### 229 Recombinant DNA Constructs

230 Full-length mouse Arc cDNA (NM\_018790.3) in pCMV-SPORT7 vector was purchased from  
 231 Open Biosystems (Huntsville, AL) and used as a template to generate the Arc constructs.  
 232 The pGFP-Arc plasmid was generated by cloning the Arc full length sequence as an  
 233 EcoRI/Sall fragment into the pEGFP-C2 vector (Clontech). Site-directed mutagenesis  
 234 (QuickChange II kit Qiagen) was used to mutate the tryptophan 197 to alanine in the  
 235 pEGFP-Arc<sub>(WT)</sub> construct. To generate constructs encoding Arc195-199A, a synthetic cDNA  
 236 sequence was obtained from GenScript (Piscataway, NJ), encoding the mouse Arc residues  
 237 1 to 700, in which codons to residues 195 to 199 (residues QSWGP) of the original Arc  
 238 sequence were replaced by codons to alanine (QSWGP/AAAAA). The Arc195-199A mutant  
 239 sequence was then used to replace the corresponding sequence in pGFP-Arc<sub>WT</sub>, using  
 240 EcoRI and a naturally occurring BglII (nt 647-652) restriction sites. This generated the pGFP-  
 241 Arc<sub>(W197A)</sub> and the pGFP-Arc<sub>(195-199A)</sub> plasmids, respectively. The plasmids encoding untagged  
 242 Arc and Arc fused to mCherry (WT and mutants) were obtained by inserting the Arc cDNAs  
 243 from pEGFP plasmids as EcoRI/Sall fragments into the pCIneo (Promega) or the pmCherry-  
 244 C2 vectors (Clontech), respectively. To express Arc and Arc mutants in *E. coli* the full-length

245 Arc (WT), Arc 1-194 and Arc 1-199 sequences were amplified by PCR with specific primers  
 246 and cloned into the pET28a vector using EcoRI and Sall restriction sites. The resulting  
 247 plasmids encode Arc fused to a hexahistidine tag at the N-terminus. To express GST-Arc<sub>(WT)</sub>  
 248 GST-Arc<sub>(195-199A)</sub> and GST-Arc<sub>(W197A)</sub> fusion proteins in *E. coli*, the Arc-WT coding sequences  
 249 in pEGFP-C2 were subcloned into pGEX5.1 (GE Healthcare) as EcoRI/Sall inserts. The  
 250 pcDNA3.1-μ2-mCherry vector was used to express μ2-adaptin in rescue experiments. This  
 251 construct was generated using a two-step cloning strategy. Firstly, cDNA encoding mouse  
 252 μ2 was amplified from pGADT7-μ2 (Guo et al., 2013) and used to replace the Leucine  
 253 Zipper (LZ) sequence, in a pcDNA3.1-based plasmid consisting of a LZ sequence followed  
 254 by a linker and the C-terminal (VC: 159-239) fragment of Venus YFP, provided by Dr  
 255 Stephen Michnick (MacDonald et al., 2006). This construct was subsequently used to  
 256 replace the VC sequence by the mCherry sequence, thus generating pcDNA3.1-μ2-  
 257 mCherry. To obtain the GFP-tagged Dynamin2 (WT) construct the open reading frame of  
 258 dynamin 2 was cloned into pEGFP-N1 as a HindIII and EcoRI insert. The pEGFP-C3 based  
 259 plasmid encoding GFP-Triad3A was previously described (Mabb et al., 2014). All open  
 260 reading frames were verified by nucleotide sequence analysis.

#### 261 Recombinant protein expression and GST-pull-down assays

262 The four subunits of rat AP-2 complex comprising residues 1 to 621 from αC adaptin (α-  
 263 trunk) fused to glutathione-S-transferase (GST) at the N-terminus, residues 1 to 591 from β2  
 264 adaptin fused to a hexahistidine tag at the C-terminus, and the full length μ2 and σ2 adaptin;  
 265 (hereafter referred to as AP2 core) were co-expressed in *E. coli* BL21 Rosetta (DE3) cells  
 266 from a pST39 vector (Sheffield et al., 1999) under the control of T7 promoter with each gene  
 267 having its own ribosome-binding site (Chaudhuri et al., 2007; Chaudhuri et al., 2009). For  
 268 GST-AP-2 core expression, bacteria were grown at 37°C to an optical density at 600 nm of  
 269 0.8. Then cultures were shifted to 18°C and the expression was induced with 0.2 mM IPTG  
 270 (isopropyl-β-D-thiogalactopyranoside) for 12 hours. The cell pellet was re-suspended in ice-  
 271 cold lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 10% glycerol, 2 mM EDTA, 10 mM

272 DTT), supplemented with 500 µg/ml lysozyme and 1 mM 4-(2-Aminoethyl) benzenesulfonyl  
 273 fluoride hydrochloride (AEBSF) and disrupted by sonication. Insoluble material was removed  
 274 by centrifugation and the AP-2 core in the supernatant was purified using a His-trap column  
 275 (GE Healthcare). Briefly, the AP-2 core complex was bound to the His-trap column via the  
 276 6xHis-β2 subunit, repeatedly washed with Tris-buffer solution (TBS) composed of 50 mM  
 277 Tris-HCl [pH 7.4], 500 mM NaCl supplemented with 30 mM of imidazol (Sigma) and eluted  
 278 with TBS with 0.25 M of imidazol. Recombinant GST (pGEX plasmid), GST-Arc<sub>(WT)</sub>, GST-  
 279 Arc<sub>(195-199A)</sub>, GST-Arc<sub>(W197A)</sub> and 6XHis-Arc (wild-type and truncated) were also expressed in  
 280 *E. coli* BL21 Rosetta (DE3) cells at 30°C with 0.5 mM IPTG. The pellet was re-suspended in  
 281 ice-cold lysis buffer, sonicated and after centrifugation, supernatant containing the soluble  
 282 proteins was used for pull-down assays.

283 Recombinant GST-AP-2 core or GST alone was immobilized onto glutathione-sepharose  
 284 beads (GE Healthcare) overnight at 4°C. Beads were washed with ice-cold TBS containing  
 285 5% of Triton X-100 (Sigma) and incubated with either His-trap column purified 6xHis-Arc or  
 286 total cell lysates of *E. coli* expressing 6x-His-Arc proteins for three hours. After four washes  
 287 with ice-cold TBS plus 5% of Triton X-100 the beads were re-suspended in sample buffer  
 288 (SDS 4%, Tris-HCl 160 mM [pH 6.8], glycerol 20%, DTT 100 mM and bromphenol blue  
 289 0.005%), boiled and proteins were separated by SDS-PAGE and transferred onto a  
 290 nitrocellulose membrane (GE Healthcare), which were then blocked for one hour with PBS,  
 291 0.1% tween 20 and 5% milk powder. Primary mouse monoclonal anti-His tag antibody  
 292 (1:1000 dilution, Sigma H1029) were added in PBS, 1% BSA for one hour. After three  
 293 washes with PBS-T, the membranes were incubated with HRP-conjugated secondary  
 294 antibody for 1 h and washed again.

295 Recombinant GST, GST-Arc<sub>(WT)</sub>, GST-Arc<sub>(W197A)</sub> and GST-Arc<sub>(195-199A)</sub> were immobilized onto  
 296 glutathione-sepharose beads overnight at 4°C. Beads were incubated with either total brain  
 297 tissue lysate, obtained as described earlier for hippocampi lysate, or total lysates of HEK293  
 298 cells expressing either dynamin 2-GFP or GFP-Triad3A for 1 h at 4 °C on ice. The beads  
 299 were centrifuged at 100 × g, washed three times with lysis buffer, supplemented with 1%

300 (v/v) Triton X-100, and subsequently resuspended in SDS-PAGE sample buffer. Beads were  
301 boiled, and proteins were resolved by SDS-PAGE and analyzed by immunoblot as described  
302 above using mouse monoclonal anti-AP-50  $\mu 2$  subunit (1:500 dilution; BD 611350) , anti- $\alpha$ -  
303 adaptin1/2 (1:1000 dilution, sc-17771) and rabbit polyclonal anti-GFP antibodies. Proteins  
304 were detected using ECL reagents.

#### 305 Immunocytochemistry

306 H4 neuroglioma cells (ATCC) were transfected with plasmids encoding a myc-tag at the N-  
307 terminus of GluA1 (Leuschner and Hoch, 1999) together with plasmids encoding either  
308 mCherry, mCherry-Arc-WT or mCherry-Arc<sub>(W197A)</sub>. 20 h after transfection, cells were fixed  
309 using 4% paraformaldehyde (PFA, pH 7.4) in 0.1 M phosphate-buffered saline (PBS) for 15  
310 min at room temperature and incubated for 30 min at 37°C in blocking solution (0.2% pork  
311 skin gelatin) in PBS. Cells were then incubated with hybridoma culture supernatant (9E10)  
312 containing mouse monoclonal anti-myc antibody (at 1:10 dilution). Cells were washed with  
313 PBS and incubated with Alexa Fluor 488 anti-mouse IgG (1:1000; Life technologies) diluted  
314 in blocking solution. Cells were then permeabilized for 10 min with 0.1% Triton X-100 in PBS  
315 and incubated again with rabbit polyclonal anti-myc antibody (a gift from R Hegde, MRC,  
316 LMB, Cambridge, UK) for 30 min at 37°C in blocking solution. This was followed by  
317 incubation with Alexa Fluor 647 anti-rabbit IgG (1:1000; Life technologies) diluted in blocking  
318 solution. Coverslips were mounted on glass slides, and cells were imaged using a confocal  
319 laser-scanning microscope (LSM) using a Zeiss LSM 780 confocal microscope (Zeiss, Jena,  
320 Germany).

321

#### 322 Biotinylation assays

323 To analyze the amount of surface and intracellular GluA1 and GluA2 proteins H4  
324 neuroglioma cells were transfected and subject to a biotinylation protocol previously  
325 described (Eales et al., 2014). Briefly, the same amount of H4 cells were seeded in each  
326 well of six wells dishes ( $3 \times 10^5$  cells/well) and then transfected with 2  $\mu$ g of plasmids encoding

327 N-terminus-myc-tagged GluA1 or GluA2 (Leuschner and Hoch, 1999) in combination with 2  
 328  $\mu$ g of pCIneo, pCIneo-Arc<sub>(WT)</sub> or pCIneo-Arc<sub>(W197A)</sub> using lipofectamine 2000. After 24 hours  
 329 the cells were washed and incubated with 1 ml of 0.25 mg/mL of EZ-Link Sulfo-NHS-SS-  
 330 Biotin (Thermo Scientific) in ice-cold PBS for 15 min at 4 °C. The cells were washed twice  
 331 with ice cold PBS, with 3 mL of NH<sub>4</sub>Cl 50 mM for 5 minutes (4°C on a shaker) and then  
 332 once more with PBS. After washing, cells were lysed with 100  $\mu$ l of lysis buffer (described  
 333 above) containing protease inhibitors, rotated for 1 hour at 4°C, centrifuged at 20.000 g for  
 334 10 minutes at 4°C and the supernatants collected. The protein concentration was assayed  
 335 using the BioRad Protein Assay Reagent and equal amounts were incubated with pre-  
 336 washed 30  $\mu$ l of NeutrAvidin® Ultra-link Resin (Life Technologies) for 3 hours on a wheel at  
 337 4°C. The beads were washed 3 times with lysis buffer, and the proteins eluted from the  
 338 beads using 20  $\mu$ l of 5x loading buffer. Proteins were loaded on an 8% SDS-PAGE gel. The  
 339 input represents 1% of the total protein incubated with the beads. The Western blot was  
 340 performed as described above.

#### 341 Lentiviruses production

342 A lentiviral transduction system was used to achieve efficient delivery of specific microRNA-  
 343 adapted shRNA sequences into neurons. Double-stranded oligonucleotides encoding  
 344 shRNAs targeting the mouse  $\mu$ 2 subunit (shRNA1:  
 345 tgctgtgaattgccctccatattggtgtttggccactgactgacaacatattagggcaattca/  
 346 cctgtgaattgccctatattggtgtcagtcagtgccaaaacaacatattgagggaattcac; shRNA2:  
 347 tgctgcattattggtactctattgcctgtttggccactgactgacaggcaatagtaggaatattg/  
 348 cctgcattattggtattggtcctgtcagtcagtgccaaaacaggcaatagtaggaatattg; shRNA3:  
 349 tgctgatctgcaggacattgcttcacgtttggccactgactgacgtgaagcagtcctgcagat/  
 350 cctgatagattcctatcaggctggtcagtcagtgccaaaacaggcctgagtaggaatattg) were cloned into the  
 351 linearized pcDNA6.2-GW/EmGFP-miR vector (Invitrogen). The sequences were designed  
 352 using the "BLOCK-iT™ RNAi Designer" software from Invitrogen to identify sequences  
 353 specific for mouse  $\mu$ 2 that are not predicted to knock down expression of any other genes. In



354 addition, the sequences have 100% homology to the target sequence and result in target  
 355 cleavage. The vector contains flanking sequences allowing the shRNAs to be expressed and  
 356 processed analogous to endogenous shRNAs. This arrangement enables the expression of  
 357 the shRNA cassette from an RNA polymerase II promoter. In addition, emGFP is expressed  
 358 iso-cistronically from the same promoter to allow the precise identification of the transduced  
 359 cells. As a negative control, the plasmid pcDNA6.2-GW/EmGFP-miR-neg control (Invitrogen)  
 360 was used. This plasmid contains an insert that forms a hairpin structure which is processed  
 361 into mature shRNA, but is predicted not to target any known vertebrate gene  
 362 (gaaatgtactgcgcgtggagacgttttgccactgactgacgtctccacgcagtacatt). The above expression  
 363 cassettes were transferred into the lentiviral expression vector pLenti6/V5-DEST (Invitrogen)  
 364 by gateway cloning. Lentiviruses were produced according to the instructions of the  
 365 manufacturer (Invitrogen; Block-It HiPerform Lentiviral Pol II RNAi Expression system with  
 366 emGFP; K4934). Lentivirus particles were collected from the culture supernatants, purified  
 367 and concentrated by incubation with 8.5% PEG 6000 and 0.4M NaCl for 1.5 hours at 4°C,  
 368 followed by centrifugation at 7000 g for 10 min (4°C). Pellets were re-dissolved in  
 369 neurobasal medium.

370

#### 371 Bicuculline incubation

372 To induce a chronic increase in neuronal activity, hippocampal cultures were incubated with  
 373 bicuculline (40  $\mu$ M, Sigma Aldrich) for 48 h prior to experimental work.

374

#### 375 Electrophysiological recordings and analysis of AMPAR-mediated miniature excitatory 376 postsynaptic currents (mEPSCs)

377 Miniature excitatory postsynaptic currents (mEPSCs) were recorded from 15-18 DIV cultured  
 378 pyramidal hippocampal neurons (Mabb et al., 2014). A coverslip was transferred to the  
 379 recording chamber and perfused at a constant flow rate of (2-3 min<sup>-1</sup>) with a recording  
 380 solution composed of (mM): 127 NaCl, 1.9 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 1.3 KH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>,  
 381 10 D-glucose, pH 7.4 (when bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, 300 mOSM) at 28-30°C. To



382 isolate AMPA receptor mediated mEPSCs, tetrodotoxin (1  $\mu$ M, Tocris), picrotoxin (50  $\mu$ M,  
 383 Sigma-Aldrich) and L-689,560 (5  $\mu$ M, Tocris) were present in the recording solution.  
 384 Cultured neurons were visualized using IR-DIC optics with an Olympus BX51W1 microscope  
 385 and Hitachi CCD camera (Scientifica, Bedford UK). Whole-cell patch clamp recordings were  
 386 made from transfected (identified by fluorescence at 488 nm) and neighboring untransfected  
 387 pyramidal neurons with patch pipettes (5-8 M $\Omega$ ) made from thick walled borosilicate glass  
 388 (Harvard Apparatus, Edenbridge UK) filled with (mM): potassium gluconate 135, NaCl 7,  
 389 HEPES 10, EGTA 0.5, phosphocreatine 10, MgATP 2, NaGTP, pH 7.2, 290 mOSM).  
 390 Recordings of mEPSCs were obtained at a holding potential of -75 mV using an Axon  
 391 Multiclamp 700B amplifier (Molecular Devices, USA), filtered at 3 kHz and digitized at 20  
 392 kHz (Digidata 1440A, Molecular Devices). For rectification experiments, the intracellular  
 393 solution contained (mM): CsCl 135, HEPES 10, EGTA 10, Mg-ATP 2, spermine 0.1; pH 7.2  
 394 with tetraethylammonium-OH (TEA-OH); 285 mOSM. To calculate the rectification index,  
 395 mEPSC recordings were made at holding potentials of -60 mV and +40 mV. Data acquisition  
 396 was performed using pClamp 10 (Molecular Devices).  
 397 Analysis of mEPSCs was performed using MiniAnalysis software (SynptoSoft, Decatur,  
 398 GA). For most experiments, where the holding potential was -75 mV, events were manually  
 399 analyzed and were accepted if they had an amplitude >6 pA and a faster rise than decay.  
 400 For the rectification experiments, where the holding potential was -60 and +40 mV and thus  
 401 mEPSCs had a smaller amplitude, events were accepted if they had an amplitude > 3 pA  
 402 and a waveform with a faster rise than decay. Cumulative probability curves for mEPSC  
 403 amplitude were constructed from 1000-2000 mEPSCs pooled from all recordings, with the  
 404 same number of mEPSCs (150) measured from each recording (Origin, Microcal). The  
 405 interval between events was measured using MiniAnalysis software. To measure mEPSC  
 406 kinetics, mEPSCs within individual recordings were aligned on the half-amplitude of their rise  
 407 and averaged (50-100 mEPSCs were averaged in each recording). The decay of the mean  
 408 current from each recording was fitted with a single exponential (maximum likelihood,  
 409 MiniAnalysis or Microcal Origin). Rise times were measured from mean currents as the time

410 required for the current to rise from 10 to 90 % of peak amplitude. The rectification index was  
 411 calculated for each recording (peak amplitude at +40 mV divided by peak amplitude at -60  
 412 mV) and then the mean rectification index was calculated for each experimental connection.  
 413 For each cell an average of 100-200 mEPSCs were analyzed. Individual mEPSCs were  
 414 aligned to 50 % of the rise, averaged and then the mean amplitude was measured from the  
 415 peak of this mEPSC waveform. Statistical significance was measured using the Mann-  
 416 Whitney test. Where possible, comparisons were made between transfected and  
 417 untransfected neighboring neurons in the same culture. For each experimental condition  
 418 cells were recorded and analyzed using hippocampal cultures from 4-5 different  
 419 preparations.

420

#### 421 Statistical Analysis

422 Data was analyzed using Prism (Version 5.04, GraphPad) and Statistical Package for the  
 423 Social Sciences 21 (IBM) software. Mann-Whitney t-tests, Kolmogorov-Smirnov test, one-  
 424 way ANOVA and the corresponding post-hoc tests (Tukey or Dunn's) were performed as  
 425 appropriate.

426

#### 427 Results

##### 428 Arc interacts with the AP-2 complex in neurons

429 Arc has been shown to regulate glutamatergic synaptic transmission by dynamically  
 430 enhancing AMPAR endocytosis in postsynaptic neurons (Shepherd et al., 2006; Mabb et al.,  
 431 2014). Given the importance of Arc in facilitating AMPAR endocytosis during synaptic  
 432 transmission we speculated that it may play a decisive role in selecting the cargo to be  
 433 internalized. To test whether Arc interacts with proteins of the clathrin-mediated endocytic  
 434 (CME) machinery and whether Arc is involved in selecting the cargo to be targeted for  
 435 endocytosis, we used the specific rabbit anti-Arc antibody to immunoprecipitate (IP)  
 436 endogenous Arc from adult C57BL6/J mice hippocampal lysate combined with mass  
 437 spectrometric analysis to identify novel Arc binding partners. The control for the IP was

438 obtained by incubating hippocampal lysate protein with the G-agarose beads in the absence  
 439 of Arc antibody. The eluted proteins from both Arc-IP and control-IP samples were subjected  
 440 to tandem mass spectrometry analysis. We only considered peptides present in the Arc-IPs  
 441 for further analysis and discarded unspecific peptides present in both Arc- and control-IPs.  
 442 Using this criteria we identified different subunits of the adaptor protein complex-2 (AP-2) as  
 443 endogenous binding partners of Arc, including the two  $\alpha$  adaptin isoforms:  $\alpha$  also known as  
 444  $\alpha$ A (19 peptides and recovery of 22.83%; NP\_031484) and  $\alpha$ 2, also known as  $\alpha$ C (11  
 445 peptides and recovery of 12.37%; NP\_031485) as well as  $\beta$ 2 (11 peptides and recovery of  
 446 12.38%; NP\_082191) and  $\mu$ 2 (9 peptides and recovery of 20.79%; Q3TWV4). These  
 447 peptides were found independently in two experimental repeats. We also found clathrin  
 448 heavy chain (30 peptides and recovery of 20%; NP\_001003908), dynamin 1 (10 peptides  
 449 and recovery of 10.57%; NP\_034195), CamKII  $\beta$  subunit (9 peptides and recovery of  
 450 20.48%, NP\_031621) and PSD95 (2 peptides and recovery 5.77%, NP\_031890).  
 451 Importantly, PSD95, dynamin and CamKII $\beta$  have previously been shown to co-IP with Arc  
 452 (Lyford et al., 1995; Chowdhury et al., 2006; Okuno et al., 2012). To further confirm that Arc  
 453 interacts with AP-2 endogenously, we immunoprecipitated Arc protein from hippocampal  
 454 lysate as previously described and resolved the proteins using SDS-PAGE. Immunoblot  
 455 analysis confirmed that Arc co-immunoprecipitates with the  $\alpha$  subunit of the AP-2 complex  
 456 (Fig. 1a,b). We observed that clathrin heavy chain also co-immunoprecipitates with Arc (Fig.  
 457 1b). This result was expected as clathrin heavy chain is known to interact with AP-2 (ter  
 458 Haar et al., 2000; Edeling et al., 2006; Knuehl et al., 2006). Together these findings suggest  
 459 an interaction between Arc and the proteins of the CME machinery that are responsible for  
 460 selecting the cargo to be internalized. To test whether Arc directly interacts with AP-2, we  
 461 performed *in vitro* GST-pull-down assays using recombinant forms of Arc-wild-type (Arc-WT)  
 462 and AP-2. Previous studies used recombinant AP-2 “core” complexes to demonstrate the  
 463 direct interaction between AP-2 and the cytosolic tail of transmembrane cargo proteins  
 464 (Höning et al., 2005) or the HIV-1 accessory protein, Nef (Chaudhuri et al., 2007; Lindwasser

et al., 2008; Chaudhuri et al., 2009). Therefore, we produced recombinant Arc-WT fused to a hexahistidine tag and recombinant GST-tagged AP-2 core, comprising the N-terminal “trunk” domains of  $\alpha$  and  $\beta 2$  subunits, plus the full-length  $\mu 2$  and  $\sigma 2$  subunits in *E. coli*. The recombinant AP-2 core complex and Arc proteins were affinity purified (Fig. 1c) and used to show that GST-tagged AP-2 core binds mouse Arc-WT, as detected by immunoblot analysis (Fig. 1d). We then used the same GST-pull-down approach to map the region of Arc that interacts with AP-2. Our initial experiments demonstrated that a C-terminal fragment of Arc comprising residues 155 to 396 is sufficient to mediate the interaction with AP-2. We then tested whether Arc mutants bearing cumulative C-terminal deletions of 40 amino acid residues would retain the capacity to bind AP-2. Using this approach we showed that the Arc C-terminus (residues 200-396, Fig. 1e) is not essential for the Arc-AP-2 interaction, as truncated Arc missing these residues (Arc1-199) was still able to interact with AP-2 (Fig. 1f). Interestingly, deletion of a further five amino acid residues from the C-terminus of Arc (Arc1-194) was sufficient to prevent AP-2 binding (Fig. 1g). Binding of Arc recombinants to GST alone was negligible, thus confirming the specificity of the Arc/AP-2 interactions (Fig. 1d,f,g). Together these results demonstrated a direct and specific interaction of Arc with the fully assembled AP-2 core complex.

482

#### Conservative tryptophan 197 mediates the Arc/AP-2 interaction

Our GST-pull-down experiments indicate that the Arc<sub>195QSWGP199</sub> amino-acid sequence is required for its interaction with AP-2. Therefore, we reasoned that a single substitution of the highly conserved tryptophan in position 197, may compromise the Arc/AP-2 interaction. To test this, we performed in vitro protein binding experiments using immobilized recombinant GST-Arc<sub>(WT)</sub>, GST-Arc<sub>(195-199A)</sub> or GST-Arc<sub>(W197A)</sub> fusion proteins to pull-down the endogenous  $\alpha$  or  $\mu 2$  subunit of AP-2 from total brain tissue lysates. We detected a robust interaction between GST-Arc<sub>(WT)</sub> and  $\mu 2$  (Fig. 2a). However this interaction was dramatically reduced when GST-Arc<sub>(W197A)</sub>, Arc<sub>(195-199A)</sub> or GST alone were used as bait (Fig. 2a), indicating that W197 is crucially involved in the interaction with AP-2. It was previously shown that Arc

493 interacts with dynamin-2 and that an internal deletion of 195-214 aa in Arc disrupt this  
 494 interaction (Chowdhury et al., 2006). To test the capacity of Arc<sub>(W197A)</sub> to interact with  
 495 dynamin, we performed similar *in vitro* binding analyses using immobilized GST-Arc<sub>(WT)</sub> or  
 496 GST-Arc<sub>(W197A)</sub> to pull-down GFP-dynamin-2 from HEK293 cell lysates. We confirmed that  
 497 Arc<sub>(WT)</sub> binds to dynamin, however there is a significant reduction in the interaction between  
 498 Arc<sub>(W197A)</sub> mutant and dynamin (Fig. 2b). In contrast, the Arc mutants carrying alanine  
 499 substitutions in the AP-2 binding motif still interact with the RING domain of the ubiquitin  
 500 ligase Triad3A/RNF216 (Fig. 2c), a protein recently described to interact with Arc (Mabb et  
 501 al., 2014). Binding of Arc<sub>(W197A)</sub> and Arc<sub>(195-199A)</sub> to Triad3A indicates that these alanine  
 502 mutations do not cause gross conformational changes in Arc which could prevent protein-  
 503 protein interaction.

504

#### 505 Arc-mediated internalization of GluA1 requires the Arc/AP-2 interaction

506 Arc<sub>(WT)</sub> overexpression in hippocampal neurons reduces surface levels of AMPAR by  
 507 selectively enhancing endocytosis. We reasoned that Arc-mediated endocytosis of AMPAR  
 508 may be linked to its ability to interact with the endocytic adaptor AP-2. To test this, we co-  
 509 expressed myc-GluA1 with either Arc<sub>(WT)</sub> or the Arc<sub>(W197A)</sub> mutant in H4 human neuroglioma  
 510 cells, and performed biotinylation assay to monitor GluA1 and GluA2 surface expression  
 511 levels. As previously shown in hippocampal neurons (Chowdhury et al., 2006),  
 512 overexpression of Arc-WT in H4 cells resulted in a significant reduction of myc-GluA1  
 513 surface expression levels (Fig. 3a). Importantly, the reduction in myc-GluA1 surface  
 514 expression was blocked when Arc<sub>(W197A)</sub> mutant, that does not bind AP-2, was co-expressed  
 515 with myc-GluA1 (Fig. 3a). Interestingly no changes in GluA2 surface expression were  
 516 observed when myc-GluA2 construct was co-expressed with either Arc<sub>(WT)</sub> or the Arc<sub>(W197A)</sub>  
 517 mutant (Fig. 3b), indicating that the GluA2 subunit is potentially less sensitive to Arc than  
 518 GluA1 as previously suggested by Chowdhury et al. (2006). To test whether Arc  
 519 overexpression induces general endocytosis of AP-2/clathrin cargo proteins, we examined  
 520 the surface levels of EGF receptor (EGFR) in H4 cells expressing either Arc-WT or the

521 Arc<sub>(W197A)</sub> mutant. As expected, expression of Arc has no significant effect in surface  
 522 expression of EGFR (Fig. 3b). To confirm whether Arc<sub>(W197A)</sub> mutant had an impact on the  
 523 Arc-dependent internalization of GluA1, we used the same experimental condition described  
 524 above to perform immunocytochemistry to label the amount of n terminus-myc-tagged GluA1  
 525 expressed at the surface. Confocal microscopy analyses confirmed that Arc<sub>(WT)</sub>  
 526 overexpression promotes a significant reduction of the GluA1 expression at the cell surface,  
 527 an effect that is impaired in cells expressing the Arc<sub>(W197A)</sub>, that cannot bind to AP-2 (Fig. 3c-  
 528 g). Together, these results indicate that Arc/AP-2 interaction is required to facilitate AMPAR  
 529 internalization.

530

# 531 The Arc/AP-2 interaction regulates AMPAR-mediated synaptic currents

532 Previous findings have demonstrated that under basal conditions hippocampal cultured  
 533 neurons overexpressing Arc-WT have significantly less AMPAR on their surface than  
 534 neighboring untransfected neurons (Shepherd et al., 2006). There is also a significant  
 535 reduction in the amplitude of AMPAR-mediated synaptic currents in CA1 neurons  
 536 overexpressing Arc-WT protein in hippocampal slices (Rial Verde et al., 2006). Conversely,  
 537 cultured hippocampal neurons from Arc knockout mice exhibit an increased density of  
 538 AMPAR at the cell surface and a deficit in AMPAR endocytosis (Chowdhury et al., 2006).  
 539 Since Arc facilitates endocytosis of AMPAR and we have demonstrated that Arc directly  
 540 binds to the AP-2 complex, we predicted that the Arc/AP-2 interaction regulates expression  
 541 of synaptic AMPAR. To test our prediction, we first recorded AMPAR-mediated mEPSCs  
 542 from cultured hippocampal neurons overexpressing an Arc-WT-GFP-tagged construct and  
 543 from untransfected neighboring cells in the same cultures. This approach was used to  
 544 negate any variations in AMPAR expression which may arise from differences in cell density.  
 545 Recordings from cells expressing EGFP alone were used as a control for transfection. In  
 546 agreement with previous studies, a significant decrease in mEPSC amplitude was observed  
 547 in cells over-expressing Arc<sub>(WT)</sub> compared to untransfected neighboring cells (Fig. 4a(i)); Rial  
 548 Verde et al., 2006; Shepherd et al., 2006). Examination of the amplitude probability curves

549 from Fig. 4a shows that the majority of AMPAR-mediated mEPSCs had smaller amplitudes  
550 in the cell where Arc<sub>(WT)</sub> was overexpressed (peak shifted to the left, red trace) compared to  
551 the untransfected neighboring cell (black trace). In contrast, there was no significant  
552 difference in the amplitude of mEPSCs recorded in an eGFP-expressing cell and its  
553 untransfected neighbor (Fig. 4b(i)).

554 To test whether the Arc-mediated reduction in the AMPAR mEPSC amplitude is dependent  
555 on an interaction with AP-2, we recorded mEPSCs from hippocampal cultures  
556 overexpressing either Arc<sub>(195-199A)</sub>- or Arc<sub>(W197A)</sub>- GFP-tagged mutant constructs. As predicted,  
557 the reduction in AMPAR-dependent mEPSC amplitudes observed in cells overexpressing  
558 Arc<sub>(W197A)</sub> or Arc<sub>(195-199A)</sub> was significantly less pronounced when compared to cells  
559 overexpressing Arc<sub>(WT)</sub> (Fig. 4c,d(i)). Pooled data is displayed as cumulative probability  
560 distributions (Fig. 4e) and as bar charts plotting the mean amplitude and interval (Fig. 4f-g).

561 Our biochemical data shows that Arc preferentially internalises GluA1 rather than GluA2  
562 subunits (Fig. 3a-b). To test whether Arc has similar effects on the endogenous AMPA  
563 receptors, which are expressed at synapses, we measured the rectification of AMPA  
564 receptor mediated mEPSC amplitudes. The reduction in the surface expression of synaptic  
565 AMPA receptors containing GluA1 subunits would be expected to reduce rectification at  
566 positive holding potentials (Bowie and Mayer, 1995; Kamboj et al., 1995; Plant et al., 2006).  
567 As predicted, the rectification index (calculated by dividing the amplitude of mEPSCs at +40  
568 mV by the amplitude at -60 mV) was significantly increased in cells expressing Arc<sub>(WT)</sub> when  
569 compared to GFP and Arc<sub>W197A</sub> expressing cells (Fig. 4h-i). Neither mEPSC rise or decay  
570 kinetics were significantly effected by overexpression of Arc<sub>(WT)</sub>, Arc mutants or eGFP (Fig.  
571 5a-d). The consistency in mEPSC rise and decay kinetics across recordings (Fig. 5c-d)  
572 demonstrates that any changes in mEPSC amplitude are a result of receptor internalisation  
573 rather than variations in recording quality. These experiments suggest that the reduction in  
574 mEPSC amplitude induced by Arc<sub>(WT)</sub> overexpression in hippocampal neurons is dependent  
575 on the binding of Arc<sub>(WT)</sub> to the AP-2 complex.

576



577 The AP2 subunit  $\mu 2$  is required for the Arc<sub>WT</sub>-induced reduction in mEPSC amplitude

578 Previous studies have shown that depletion of the  $\mu 2$  subunit compromises the stability of  
579 the remaining subunits of AP-2 and also that the complexes lacking the  $\mu 2$  subunit are  
580 inactive and fail to localize to the plasma membrane (Meyer et al., 2000; Peden et al., 2002;  
581 Motley et al., 2003). To further investigate the importance of the Arc/AP-2 interaction, we  
582 designed shRNA-like sequences to knockdown the endogenous expression of  $\mu 2$  in mouse  
583 tissue. We then used these shRNA constructs to transfect the mouse cell line NSC-34. A  
584 shRNA sequence, not predicted to knockdown any vertebrate genes, was used as a  
585 negative control. Using this approach we identified two out of three shRNA sequences ( $\mu 2$ -  
586 shRNA<sub>2</sub> and  $\mu 2$ -shRNA<sub>3</sub>) that efficiently reduced the protein expression of  $\mu 2$  in NSC-34  
587 cells (Fig. 6a). To knockdown endogenous  $\mu 2$  in neurons, we generated lentiviruses  
588 expressing these two shRNAs. The lentiviruses also express emGFP isocistronically, to  
589 efficiently identify the transduced neurons. Lentiviral transduction of  $\mu 2$ -shRNA<sub>2</sub> into  
590 hippocampal cultures resulted in an overall 50% reduction in  $\mu 2$  expression compared to the  
591 negative control shRNA (Fig. 6b). Note that even under optimal circumstances transduction  
592 rates in primary neurons are between 70-80% using lentiviral systems. This indicates that a  
593 significantly more pronounced reduction in  $\mu 2$  expression has been achieved in those cells  
594 that have been transduced and used for recordings. To examine whether AP-2 is required in  
595 AMPAR-mediated synaptic transmission under basal conditions, we first transduced  
596 hippocampal cultures at 6-7 DIV with a lentivirus expressing  $\mu 2$ -shRNA<sub>2</sub>-emGFP and  
597 recorded AMPAR-mediated mEPSCs 7-8 days after transfection. No significant change in  
598 mEPSC amplitude was observed in cells expressing  $\mu 2$ -shRNA<sub>2</sub> alone compared to  
599 untransfected neighboring cells (Fig. 6c(i)). These findings suggest that the constitutive  
600 endocytosis of AMPAR occurring under basal conditions in cultured hippocampal neurons is  
601 not strictly dependent on AP-2.

602 To test whether AP-2 is required for Arc-mediated endocytosis of AMPAR, we recorded  
603 mEPSCs from hippocampal neurons expressing either  $\mu 2$ -shRNA<sub>2</sub>-emGFP- plus mCherry-  
604 Arc-WT or the negative control (n.c.) shRNA-emGFP plus mCherry-Arc-WT as well as



untransfected neighboring neurons. Consistent with our hypothesis, a 30% reduction in mEPSC amplitudes was seen in neurons expressing Arc<sub>(WT)</sub> plus n.c. shRNA (Fig. 6d(i)). However this reduction in mEPSC amplitude was abolished in cells co-expressing Arc-WT plus  $\mu$ 2-shRNA<sub>2</sub> (Fig. 6e(i)). Pooled data is displayed as cumulative probability distributions (Fig. 6f) and as bar charts plotting the mean amplitude and interval (Fig. 6g,h). To confirm this observation, we also recorded AMPAR-mediated mEPSCs from neurons expressing either  $\mu$ 2-shRNA<sub>3</sub> alone or together with Arc-WT. Again, no change in mEPSC amplitudes was seen in cells expressing  $\mu$ 2-shRNA<sub>3</sub> alone (Fig. 6i,j). However, expression of  $\mu$ 2-shRNA<sub>3</sub> blocked the Arc-WT-mediated decrease in mEPSC amplitudes (Fig. 6i-k). Neither mEPSC rise or decay kinetics was significantly affected by overexpression of either  $\mu$ 2-shRNA<sub>2</sub> or  $\mu$ 2-shRNA<sub>3</sub> alone, Arc-WT plus  $\mu$ 2-shRNA<sub>2</sub> or  $\mu$ 2-shRNA<sub>3</sub> or Arc<sub>(WT)</sub> plus n.c. shRNA (Fig. 5). These results demonstrate that knockdown of the AP-2 complex is sufficient to disrupt the Arc-mediated endocytosis of AMPAR. Together, these findings suggest that AP-2 is required for the Arc-mediated endocytosis of AMPAR in hippocampal neurons.

#### Arc-mediated reduction in AMPAR-mediated mEPSC amplitude requires the binding of Arc to AP-2

We have shown that: 1) the reduction in AMPAR-mediated mEPSC amplitude observed in neurons overexpressing Arc<sub>(WT)</sub> is either reduced or abolished in neurons expressing mutated Arc, that cannot bind to AP-2 (Fig. 4) and 2) that the effect of Arc-WT overexpression on mEPSC amplitude is reduced in neurons expressing a decreased amount of AP-2 $\mu$ 2 protein (Fig. 6). This data suggests that Arc requires AP-2 to facilitate the internalization of AMPAR. To confirm the functional relationship between Arc and AP-2, we recorded mEPSCs from hippocampal neurons expressing Arc-WT and  $\mu$ 2-shRNA<sub>2</sub>-emGFP in the same lentivirus combined with re-expression of  $\mu$ 2 using another lentivirus expressing a  $\mu$ 2-shRNA<sub>2</sub> resistant  $\mu$ 2-mCherry fusion protein. As a control, lentiviruses encoding Arc<sub>(195-199A)</sub> $\mu$ 2-shRNA<sub>2</sub>-emGFP and  $\mu$ 2-mCherry was used to transduce hippocampal cultures. As predicted, the Arc<sub>(WT)</sub>-mediated reduction in AMPAR mEPSC amplitude caused by depletion

633 of AP-2/ $\mu$ 2 (see Fig. 4) was reversed by overexpressing  $\mu$ 2 (Fig. 7a(i)), demonstrating that  
 634 AP-2/ $\mu$ 2 is specifically required for the effect of Arc on AMPAR amplitudes. In contrast, no  
 635 effect on AMPAR amplitudes was seen in cells expressing a mutant form of Arc<sub>(195-199A)</sub> that  
 636 cannot bind to AP-2, irrespective of the expression status of  $\mu$ 2 (Fig. 7b(i), c). Pooled data is  
 637 displayed as cumulative probability distributions (Fig. 7c) and as bar charts plotting the mean  
 638 amplitude and interval (Fig. 7 d-e). Neither mEPSC rise or decay kinetics were significantly  
 639 affected by overexpression of Arc-WT- $\mu$ 2-shRNA<sub>2</sub>-emGFP plus  $\mu$ 2-mCherry and Arc<sub>(195-199A)</sub>-  
 640  $\mu$ 2-shRNA<sub>2</sub>-emGFP plus  $\mu$ 2-mCherry (Fig. 5). These experiments clearly demonstrate that  
 641 the Arc/AP-2 interaction is required for the reduction in AMPAR-mediated mEPSC  
 642 amplitudes rather than sole disruption in AP-2.

643

#### 644 AP-2 is required for Arc-dependent homeostatic scaling

645 Homeostatic scaling is the ability of neurons to sense the level of synaptic activity and  
 646 compensate for changes by modulating their excitability. For example, in response to a  
 647 prolonged increase in synaptic activity, neurons reduce synaptic strength by facilitating  
 648 endocytosis of synaptic AMPAR (downscaling). Arc, whose expression is robustly induced  
 649 by increased activity, is known to facilitate synaptic downscaling by enhancing AMPAR  
 650 endocytosis (Shepherd et al., 2006; Mabb et al., 2014). Since we have shown that AP-2 is  
 651 required for the Arc-dependent endocytosis of AMPA receptors, we hypothesized that a  
 652 reduction in AP-2 expression should impair Arc-dependent synaptic scaling. To test this, we  
 653 recorded AMPAR-mediated mEPSCs from hippocampal cultured neurons chronically treated  
 654 with bicuculline (40  $\mu$ M, 48 hr), which blocks inhibitory neurotransmission mediated by  
 655 GABA<sub>A</sub> receptors and thus increases neuronal firing. In agreement with previous studies  
 656 (Shepherd et al., 2006; Mabb et al., 2014), we observed a significant decrease in the  
 657 amplitude of AMPAR-dependent mEPSCs in cells incubated with bicuculline compared to  
 658 control cells (Fig. 8a-c). To address whether AP-2 was required for this reduction in mEPSC  
 659 amplitude, we reduced  $\mu$ 2 expression by transducing hippocampal neurons with  $\mu$ 2-shRNA<sub>2</sub>  
 660 and as a control, n.c. shRNA, for 5 days prior to bicuculline incubation. In neurons

661 expressing the n.c. shRNA, bicuculline incubation still resulted in a robust reduction in  
662 mEPSC amplitude (Fig. 8b,c). However, the reduction in mEPSC amplitude was significantly  
663 smaller in neurons expressing  $\mu 2$ -shRNA<sub>2</sub> (Fig. 8a-c). None of the treatments significantly  
664 ( $p>0.05$ ) changed the frequency of mEPSCs (Fig. 8d) or the rise or decay kinetics of  
665 mEPSCs (Fig. 5). Together, these findings support the hypothesis that the Arc/AP-2  
666 interaction is required for the endocytosis of AMPAR during homeostatic scaling.

667

## 668 **Discussion**

669 The present study identifies a functional link between Arc and the AP-2 complex, a vital  
670 component of clathrin-mediated endocytic (CME) pathway. The AP-2 complex is required for  
671 selection and recruitment of the endocytic cargo and also for clathrin recruitment to the  
672 plasma membrane, processes that initiate the formation of the clathrin-coated pit (CCP)  
673 (Robinson, 2004; Saheki and De Camilli, 2012; Kelly et al., 2014; Kirchhausen et al., 2014).  
674 Here, we demonstrate that Arc immunoprecipitates with the AP-2 complex in hippocampal  
675 lysate and that Arc directly binds to the AP-2 complex (Fig. 1). We also show that the Arc  
676 residues <sub>195</sub>QSWGP<sub>199</sub> mediate the Arc/AP-2 association and that a conserved tryptophan  
677 residue at position 197 (W<sub>197</sub>) is essential for this interaction (Fig. 2). Importantly, the GST-  
678 Arc mutants that is impaired in AP-2 binding still bound to another binding partner, Triad3A,  
679 demonstrating the structural integrity of the mutated Arc proteins. Interestingly, the mutated  
680 Arc proteins pulled down higher levels of Triad3A compared with GST-Arc<sub>(WT)</sub> from cell  
681 extracts (Fig. 2). While the reasons for these results were not addressed here, one possible  
682 explanation is that preventing the AP-2 interaction may render Arc's C-terminal domain more  
683 accessible to make contacts with Triad3A, leading to increased binding. Importantly, this  
684 apparently higher affinity for the ubiquitin ligase Triad3A does not cause changes in the  
685 expression/stability of the Arc mutants (Fig. 3). This further demonstrates that the observed  
686 functional changes of the Arc mutants are specifically due to the loss of its binding to AP-2.  
687 In agreement with previous studies (Shepherd et al., 2006; Waung et al., 2008), we showed  
688 that overexpression of Arc strongly reduces surface expression of GluA1, but not GluA2 in

689 H4 neuroglioma cells (Fig. 3). In cultured hippocampal neurons, overexpression of Arc  
 690 reduces the number of synaptic AMPA receptors, as shown by a decrease in the amplitude  
 691 of AMPAR-mediated mEPSCs and also regulates AMPA receptor subunit composition (Fig.  
 692 4). It has been previously shown that AMPA receptors containing GluA2 subunits show a  
 693 linear current-voltage relationship in contrast to GluA2-lacking receptors that show  
 694 pronounced rectification (Isaac et al., 2007). In our experiments, mEPSCs recorded from  
 695 neurons overexpressing GFP alone showed pronounced rectification, suggesting that the  
 696 predominant combination of AMPA receptors lacks the GluA2 subunit (also see Eales et al  
 697 2014). In contrast overexpression of Arc resulted in diminished mEPSCs rectification,  
 698 suggesting a reduction in the proportion of synaptic receptors that lack the GluA2 subunit.  
 699 These findings are in agreement with previous studies showing that there is an increase in  
 700 surface expression of GluA1, but not GluA2 subunits in hippocampal cultures obtained from  
 701 Arc knockout mice at non-stimulated conditions (Shepherd et al., 2006). Also knockdown of  
 702 endogenous Arc in hippocampal cultures resulted in increased GluA1 subunits at the surface  
 703 at non-stimulated conditions (Waung et al., 2008). Furthermore, application of DHPG (which  
 704 induces an increase in Arc translation and protein expression) to cultured hippocampal  
 705 neurons reduced rectification (Eales et al., 2014). As expected, mutation of the AP-2 binding  
 706 site in Arc or depletion of AP-2 $\mu$ 2 compromises the capacity of Arc to reduce AMPAR-  
 707 mediated mEPSC amplitudes (Figs. 4-6). Furthermore, the Arc-mediated reduction in  
 708 AMPAR mEPSC amplitudes was rescued in cells where depletion of AP-2 $\mu$ 2 was reversed  
 709 by re-introducing  $\mu$ 2 (Fig. 7). Importantly, this rescue was compromised in cells expressing a  
 710 mutated form of Arc that cannot interact with AP-2 (Fig. 7). Furthermore, disruption of the  
 711 Arc/AP-2 interaction by reducing the expression of AP-2 $\mu$ 2 also dampens the Arc-mediated  
 712 reduction in synaptic strength observed in homeostatic synaptic downscaling (Fig. 8).  
 713 Combined, these experiments demonstrate that Arc-dependent endocytosis of AMPARs  
 714 requires an interaction of Arc with AP-2. It has been recently shown that dynamin activity is  
 715 not required to reduce AMPA receptors surface levels induced by exposure to bicuculline  
 716 and potassium chloride, suggesting that homeostatic downscaling may also be induced in a

717 clathrin-independent manner (Glebov, et al., 2014). Thus we cannot discard the possibility  
 718 that AMPA receptor endocytosis via an as yet non-identified clathrin/dynamin independent  
 719 mechanism may contribute to regulate synaptic strength seen in homeostatic synaptic  
 720 downscaling.

721

722 The requirement of Arc regulating synaptic plasticity in the hippocampus is well-established  
 723 (Rial Verde et al., 2006; Bramham et al., 2010; Jakkamsetti et al., 2013; Mabb et al., 2014).  
 724 However, to utilize Arc as a potential therapeutic target, it would be beneficial to obtain its  
 725 crystal structure. During the development of this project, no information on Arc structure was  
 726 available. As we have discovered that the interaction between Arc and AP-2 depends on a  
 727 short motif in the Arc sequence (195-199), we have undertaken homology modelling studies  
 728 using the iTASSER suite (<http://zhanglab.ccmb.med.umich.edu/I-TASSER>; Roy et al., 2010)  
 729 to investigate the structural properties of this region and to obtain clues as to the structural  
 730 nature of the interaction interface. Unfortunately we were not able to obtain a model with a  
 731 reasonable confidence score. The main reason for this is that there are no other protein  
 732 structures in the databank that are sufficiently related to Arc to allow modelling by homology  
 733 approaches. Our attempts are in agreement with a recent study (Myrum et al., 2015) that  
 734 also obtained models with low scores that were deemed to be only moderately reliable. In  
 735 addition, the central region of the protein was suggested to be largely unstructured and  
 736 flexible and the area containing the AP-2 interaction motif described in this study was not  
 737 included in the models. Interestingly, another recent study (Zhang et al., 2015) has  
 738 succeeded in obtaining a partial crystal structure of Arc, demonstrating that the C-terminal  
 739 part of Arc is evolutionary similar to the Ty3/Gypsy retrotransposon and that it shows  
 740 similarity to the HIV gag protein. However, the crystal obtained does not include the N-  
 741 terminal sequences up to amino acid 206 and therefore does not include the AP-2 binding  
 742 motif. Nevertheless, as both studies (and our own modelling approach) suggested that the  
 743 AP-2 binding motif is in a flexible and at least partly unstructured region of the protein, it is

744 highly likely that this region of Arc is able to serve as a binding platform for multiple partners,  
745 including AP-2.

746

747 Arc has been shown to mediate endocytosis of AMPAR via interaction with dynamin 2 and  
748 endophilin 3, which are accessory proteins of the CME machinery (Chowdhury et al., 2006).  
749 Endophilin and dynamin are required for membrane constriction and scission of the CCV,  
750 which are late events in the CME process. Recent evidence, using mature cultured cortical  
751 neurons from distinct knockout mice where specific endophilins have been knocked out,  
752 clearly demonstrated that the assembly and early maturation events of clathrin-coated pit  
753 formation are independent of endophilin (Milosevic et al., 2011). Dynamin is recruited at late  
754 stages of endocytosis and its enrichment coincides with neck fission and release of the  
755 vesicle (Ferguson and De Camilli, 2012; Grassart et al., 2014). These findings clearly  
756 demonstrate that endophilin and dynamin do not participate in the cargo selection process.  
757 In contrast, AP-2 plays a critical role in the initiation of clathrin-mediated endocytosis, as it  
758 coordinates the cargo recruitment and selection together with clathrin recruitment and lattice  
759 assembly (Robinson, 2004; Kirchhausen et al., 2014; Kelly et al., 2014). The AP-2 complex  
760 is thought to exist in an inactive “closed” conformation in the cytosol that prevents  
761 unproductive interaction with clathrin. Binding to plasma membrane enriched  
762 phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P<sub>2</sub>] and to transmembrane cargo, triggers  
763 conformational changes in AP-2 that are necessary to allow efficient binding to clathrin and  
764 bud formation which is thought to be the dominant mechanism for the initiation of clathrin  
765 coat assembly (Kelly et al., 2014). The current model, in which Arc is able to induce clathrin-  
766 mediated AMPAR endocytosis via interaction with endophilin and dynamin, does not place  
767 Arc as a decisive player in specifically controlling excitatory synaptic transmission.  
768 Importantly, our finding that Arc directly binds to AP-2 provides the mechanistic link by which  
769 activity-dependent expression of Arc specifically facilitates endocytosis of AMPAR. We  
770 therefore suggest a refined model where neuronal excitability induces an increase in Arc  
771 protein expression in dendritic spines (Fig. 9). Newly expressed Arc then interacts with AP-2

772 and possibly increases its affinity for the cytosolic tail of AMPA receptors. Activated AP-2  
 773 initiates the formation of the clathrin-mediated pits (CMP) by coordinating the assembly of  
 774 clathrin and binding to AMPAR at the postsynaptic density. We speculate that following the  
 775 formation of CMP, Arc then binds and recruits endophilin and dynamin, which trigger fission  
 776 of the vesicle neck. Arc may not be able to simultaneously bind to AP-2 and  
 777 endophilin/dynamin. Therefore, one possible explanation is that following CMP formation,  
 778 the affinity between Arc and AP-2 is reduced, releasing Arc from the CMP. The unbound Arc  
 779 then binds and recruits endophilin and dynamin, which promotes neck fission and release of  
 780 the CCV. Alternatively, Arc binding to dynamin/endophilin may be facilitated through AP-2  
 781 interacting partners such as amphiphysin, which is able to bind to both AP-2 and dynamin  
 782 (Slepnev et al., 2000). In fact, AP-2 has been described as a major hub for recruitment of  
 783 accessory proteins to the maturing CMP (Schmid et al., 2006; McMahon and Boucrot, 2011).  
 784 Our discovery that Arc directly binds to AP-2, which in turn regulates AMPAR endocytosis,  
 785 provides the crucial mechanistic link explaining how activity-dependent expression of Arc  
 786 regulates synaptic plasticity and therefore plays a critical role in learning and memory  
 787 formation.

788

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930

# 931 **Figure Legends**

932 **Figure 1. Arc directly interacts with the AP-2 complex.**

933 (a) Arc co-immunoprecipitates with the  $\alpha$  subunit of AP-2. Hippocampal lysate was subjected  
934 to immunoprecipitation (IP) with an Arc antibody followed by immunoblot (IB) using an anti- $\alpha$   
935 adaptin antibody. Ten percent of the protein lysate used for the IP was loaded in the input  
936 lane. (b) Arc co-immunoprecipitates with clathrin in hippocampal lysate. Hippocampal lysate  
937 was subjected to immunoprecipitation (IP) with a rabbit anti-Arc or a normal rabbit anti-IgG  
938 control antibodies followed by immunoblot (IB) using an anti- $\alpha$  adaptin and an anti-clathrin

heavy chain antibodies. Ten percent of the protein lysate used for the IP was loaded in the input lane. (c-d) Pull-down assay showing the interaction of AP-2 core with mouse Arc<sub>(WT)</sub>. Recombinant affinity purified GST-AP-2 core (GST tagged  $\alpha$  subunit (residues 1-621), 6xHis tagged  $\beta$ 2 subunit (residues 1-591), full length  $\mu$ 2 and  $\sigma$ 2 subunits), was immobilized on glutathione beads (c, right panel) and incubated with recombinant affinity purified 6xHis-Arc<sub>(WT)</sub> (c, left panel). Binding of Arc protein to GST-tagged AP-2 core or GST alone was analysed by GST pull-down and SDS-PAGE, followed by Coomassie blue staining (d, left panel) or immunoblot using an anti-Arc antibody (d, right panel). (e) Schematic representation of the Arc-WT sequence showing the truncated Arc mutants used in this study. The diagram indicates coiled-coil (CC) and spectrin repeat homology (SRH) structure domain of mouse Arc. AP-2 binding site is shown in black. (f) Pull-down assay showing the interaction of AP-2 core with mouse Arc<sub>(WT)</sub> and the Arc<sub>(1-199)</sub> truncated (deletion of residues 200-396). Recombinant affinity purified AP-2 core, was immobilized on glutathione beads and incubated with lysates of E. coli expressing Arc<sub>(WT)</sub> or Arc<sub>(1-199)</sub> deletion mutant. Binding of Arc proteins to GST-tagged AP-2 or GST alone was analysed by GST pull-down and SDS-PAGE, followed by Coomassie blue staining (left panel) or immunoblot using an anti-His tag antibody (right panel). Ten percent of the recombinant proteins used for the pull-down was loaded on the input lane (Bands corresponding to Arc proteins are indicated by white asterisks). (g) Pull-down assay showing that the Arc residues 195-199 are required for the Arc/AP-2 interaction as truncated Arc<sub>(1-194)</sub> produced in E. coli lost the ability to bind immobilised recombinant GST-tagged AP-2 core.

**Figure 2: Identification of Arc motif that binds to AP-2.**

(a) Pull-down assay showing that a conserved tryptophan residue at position 197 mediates Arc/AP-2 interaction. Recombinant GST-Arc<sub>(WT)</sub>, GST-Arc<sub>(W197A)</sub>, GST-Arc<sub>(195-199A)</sub> or GST alone were produced in E. coli and immobilized on glutathione beads (bottom) and incubated with total brain tissue lysate. Binding of endogenous  $\mu$ 2 and  $\alpha$ -adaptins to GST fusion proteins was analysed by SDS-PAGE immunoblotting with anti- $\mu$ 2 (top) or anti- $\alpha$  (middle)

antibodies. Bar chart plotting analysis of the relative amount of protein bound to GST and GST-Arc<sub>(W197A)</sub> and GST-Arc<sub>(195-199A)</sub> normalized to GST-ArcWT (100%). (b) Pull-down assay showing interaction of Arc<sub>(WT)</sub> and Arc<sub>(W197A)</sub> with dynamin 2. Recombinant GST-Arc<sub>(WT)</sub>, GST-Arc<sub>(W197A)</sub> or GST alone were produced in *E. coli*, immobilized on glutathione beads (bottom) and incubated with total lysates of HEK293 cells expressing dynamin 2-GFP. Binding of dynamin 2-GFP to GST fusion proteins was analysed by SDS-PAGE immunoblotting with anti-GFP antibody (top). Bar chart plotting analysis of the relative amount of dynamin 2 bound on the beads normalized to GST-ArcWT (100%). 10% of total protein lysate used to incubate the beads was used as input. (c) Pull-down assay showing interaction of Arc<sub>(WT)</sub>, Arc<sub>(W197A)</sub> and GST-Arc<sub>(195-199A)</sub> with Triad3A. Recombinant GST proteins produced in *E. coli* and immobilized on glutathione beads (bottom) were incubated with total lysates of HEK293 cells expressing GFP-Triad3A. Binding of GFP-Triad3A to GST fusion proteins was analyzed by SDS-PAGE immunoblotting with anti-GFP antibody (top). Bar chart plotting analysis of the relative amount of Triad3A bound on the beads normalized to GST-ArcWT (100%). 10% of total protein lysate used to incubate the beads was used as input. Errors bars represent the means  $\pm$  SEM (n=3 independent experiments). \*p<0.05, \*\*p<0.005 and \*\*\*p<0.0005 using unpaired Student's t test.

### Figure 3. Arc/AP-2 interaction regulates GluA1 endocytosis

(a,b) Representative blots showing that Arc<sub>(WT)</sub>, but not the Arc<sub>(W197A)</sub> mutant, facilitates GluA1, but not GluA2 endocytosis. H4 neuroglioma cells were transfected with plasmids encoding myc-GluA1 (a) or myc-GluA2 (b) in combination with either: empty pCIneo vector, pCIneo Arc<sub>(WT)</sub> or pCIneo Arc<sub>(W197A)</sub>. Western blot band densitometry analysis showing that: (a) Arc<sub>(WT)</sub>, but not Arc<sub>(W197A)</sub>, promotes a significant reduction in surface expression of GluA1 subunits (control: 60.46  $\pm$  2.97%; Arc<sub>(WT)</sub>: 38.55  $\pm$  7.44%; Arc<sub>(W197A)</sub>: 50.18  $\pm$  8.34%. Error bars represent the means  $\pm$  SEM (n=3 independent experiments). \*p<0.05 using one-way ANOVA followed by Tukey's post-test. (b) Arc<sub>(WT)</sub> does not promote any changes in surface expression of either GluA2 subunits (control: 132.9  $\pm$  26.66%; Arc<sub>(WT)</sub>: 133.2  $\pm$  21.78%;

993 Arc<sub>(W197A)</sub>: 143.9 ± 38.43%) or EGF receptor (control: 51.35 ± 10.43%; Arc<sub>(WT)</sub>: 38.93 ±  
 994 8,66%; Arc<sub>(W197A)</sub>: 41.59 ± 8.8%). Error bars represent the means ± SEM (n=4 independent  
 995 experiments). Ten percent of the protein lysate used for incubate the beads was loaded in  
 996 the input lane. GAPDH was used as loading controls. (c-f) H4 cells co-expressing myc-  
 997 GluA1 with either mCherry construct alone (c); mCherry-Arc<sub>(WT)</sub> (d); or mCherry-Arc<sub>(W197A)</sub>  
 998 (e). Surface myc-GluA1 (non-permeabilized cells, green channel) was identified using  
 999 mouse anti-myc antibody followed by Alexa 488 secondary antibody and internal myc-GluA1  
 1000 (permeabilized, magenta channel) was identified by polyclonal rabbit anti-myc antibody  
 1001 followed by Alexa 647 secondary antibody. (f,g) The mean florescence intensity (MFI) of  
 1002 Alexa488 (surface my-GluA1) and mCherry (red channel) were calculated using confocal Z-  
 1003 projection images to quantify the pixel intensity of surface myc-GluA1 and mCherry (total  
 1004 protein expression). (f) Ratio of averaged MFI between surface (488)/total protein (mCherry)  
 1005 for control cells (n=59 cells) was set to 100 % to facilitate comparison. Note that the ratio for  
 1006 surface GluA1 is significantly reduced in cells expressing mCherry-Arc<sub>(WT)</sub> 34,68 ± 3,13%; n=  
 1007 60 cells) compared to cells expressing mCherry construct alone. Importantly, this reduction  
 1008 is absent in cells expressing the mCherry-Arc<sub>(W197A)</sub> construct (114,80 ± 13,08%; n= 42 cells.  
 1009 (g) Bar chart plotting the averaged MFI expression levels of mCherry-Arc<sub>(WT)</sub> and mCherry-  
 1010 Arc<sub>(W197A)</sub> compared to mCherry expression. Values are the mean ± SEM (n=3 independent  
 1011 experiments). \*p<0.05; \*\*\*p<0.005 using one-way ANOVA followed by Tukey's posttest.  
 1012 Scale bar=10 µm. (h) Representative blot and bar chart plotting bands densitometry analysis  
 1013 of Arc expression protein in H4 cells transfected with equal amounts of mCherry-Arc<sub>(WT)</sub>,  
 1014 mCherry-Arc<sub>(W197A)</sub>, or mCherry-Arc<sub>(195-199A)</sub> plasmids. Note the similar levels Arc protein  
 1015 expression between samples. Values are the mean ± SEM (n=3 independent experiments).

#### 1016 **Figure 4. The Arc/AP-2 interaction regulates AMPAR-mediated synaptic currents**

1017 (a-d) Representative live imaging of a dissociated hippocampal neuron overexpressing Arc-  
 1018 GFP-tagged constructs and GFP. (a) AMPAR-mediated mEPSC traces from a neuron  
 1019 overexpressing Arc<sub>(WT)</sub> and an untransfected neighboring neuron. (ai) Amplitude probability



1020 distribution for the mEPSCs shown in (a). Note the shift to the left and increase in the  
 1021 amplitude of the main peak in the neuron overexpressing Arc<sub>(WT)</sub>, clearly demonstrating the  
 1022 reduction in mEPSC amplitude. Inset, superimposed average mEPSC waveforms. (b)  
 1023 Representative AMPAR-mediated mEPSC traces from a neuron overexpressing GFP and  
 1024 an untransfected neighboring neuron. (bi) Amplitude probability distributions for mEPSCs  
 1025 recorded from the neurons shown in (b). Inset, superimposed average mEPSC waveforms.  
 1026 (c) Representative AMPAR-mediated mEPSC traces from a neuron expressing Arc<sub>(W197A)</sub>  
 1027 and an untransfected neighbouring neuron. (ci) Amplitude probability distributions from  
 1028 neurons shown in (c). Note that expression of Arc<sub>(W197A)</sub> produced a smaller reduction in  
 1029 mEPSC amplitude compared to Arc<sub>(WT)</sub> overexpression. Inset, superimposed average  
 1030 mEPSC waveforms. (d) Representative AMPAR-mediated mEPSC traces from a neuron  
 1031 expressing Arc<sub>(195-199A)</sub> and an untransfected neighboring neuron. (di) Amplitude probability  
 1032 distributions from neurons shown in (d). Note that expression of Arc<sub>(195-199A)</sub>, in which the  
 1033 sequence 195QSWGP199 of Arc was mutated to 195AAAAA199 had little effect on mEPSC  
 1034 amplitude. Inset, superimposed average mEPSC waveforms. (e) Cumulative probability  
 1035 distributions for cells expressing Arc<sub>(WT)</sub> (12 neurons), Arc<sub>(W197A)</sub> (13 neurons), Arc<sub>(195-199A)</sub> (10  
 1036 neurons), GFP (7 neurons) and for untransfected cells (20 neurons). (f), Bar chart plotting  
 1037 mean mEPSC amplitude for the cells in (e). Expression of Arc<sub>(WT)</sub> significantly reduced  
 1038 mEPSC amplitude (mean reduced from  $15.99 \pm 0.9$  pA in untransfected cells to  $10.56 \pm 0.66$   
 1039 pA,  $p=0.0002$ ) whereas expression of Arc<sub>(W197A)</sub> or Arc<sub>(195-199A)</sub> had no significant effect ( $14.6$   
 1040  $\pm 0.74$  pA,  $p=0.12$  and  $14.01 \pm 1.2$  pA,  $p=0.37$ ). Expression of eGFP had no significant  
 1041 effect ( $p=0.376$ ) on the mean mEPSC amplitude compared to untransfected cells. (g) Bar  
 1042 chart plotting the mean interval between mEPSCs. Expression of Arc<sub>(WT)</sub> and the Arc  
 1043 mutants had no significant effect on the frequency of mEPSCs. Although the mean  
 1044 frequency of mEPSCs in cells expression Arc<sub>(WT)</sub> appeared reduced, this was not significant  
 1045 as there was large variability between cells. (h) Representative average mEPSC waveforms  
 1046 recorded at a holding potential of -60 and + 40 mV for cells expressing GFP, Arc<sub>(WT)</sub> and  
 1047 Arc<sub>(W197A)</sub> in the presence of spermine (100  $\mu$ M) in the intracellular solution. (i) Bar chart

1048 plotting the mean rectification index (peak amplitude at + 40 mV divided by peak amplitude  
1049 at -60 mV) for neurons expressing GFP (n = 9 cells;  $0.34 \pm 0.015$ ), Arc<sub>(WT)</sub> (n = 9 cells;  $0.62 \pm$   
1050  $0.016$ ) and Arc<sub>(W197A)</sub> (n = 6 cells;  $0.45 \pm 0.015$ ). Thus Arc<sub>(WT)</sub> reduces the amount of  
1051 rectification (as seen as an increase in the rectification index) whereas Arc<sub>(W197A)</sub> has  
1052 significantly less effect on rectification. Error bars in f, g and i are SEMs \*\*\*p<0.001;  
1053 \*\*p<0.01. Statistical significance was tested using the Mann-Whitney test. Scale bar=10  $\mu$ m

1054 **Figure 5. Overexpression of Arc-cDNAs does not affect AMPAR-mediated mEPSC**  
1055 **kinetics in hippocampal neurons**

1056 (a) Average of 75 miniature excitatory postsynaptic currents (mEPSCs, aligned on the mid-  
1057 point of the rising phase) from an individual neuron expressing Arc<sub>(WT)</sub>. The decay was fitted  
1058 with a single exponential ( $\tau = 4.5$  ms, black line). Inset: the average mEPSC at an expanded  
1059 time-base showing the exponential fit to the decay. (b) Average of 80 mEPSCs (aligned on  
1060 the mid-point of the rising phase) from an untransfected neuron which was a close neighbour  
1061 to the cell in (A). The decay of the mEPSC was very similar to the transfected neighbour (the  
1062 decay was fitted with a single exponential;  $\tau = 4.7$  ms, black line). Inset: the average mEPSC  
1063 at an expanded time-base to show the exponential fit to the decay. (c) Bar chart plotting the  
1064 mean 10-90 % rise-time of mEPSCs recorded from untransfected neurons (n= 18) and from  
1065 neurons expressing different constructs and in different conditions (n = 6 for each). The  
1066 mean rise-time was calculated by averaging the rise-time of mean currents from individual  
1067 recordings. There was no significant difference in the mean mEPSC rise-time recorded from  
1068 any of the neurons. (d) Bar chart plotting the mean decay time constant ( $\tau$ ) from  
1069 untransfected neurons (n=18) and from neurons expressing different constructs and in  
1070 different conditions (n=6 for each). The mean decay time constant ( $\tau$ ) was calculated by  
1071 averaging the time constant from the decay of mean currents from individual recordings. The  
1072 decay of mEPSCs was not significantly different between conditions. The error bars in (c)  
1073 and (d) are SEMs. Statistical significance was tested using the Mann-Whitney test

1074 **Figure 6. AP-2 is required for Arc-dependent changes in synaptic strength**

1075 (a) Blots showing levels of  $\mu 2$  protein obtained from NSC cells overexpressing negative  
 1076 control (n.c.) shRNA,  $\mu 2$ -shRNA<sub>2</sub>,  $\mu 2$ -shRNA<sub>3</sub> plasmids for 3-4 days. GAPDH was used as  
 1077 loading control. Bar chart plotting the analysis of  $\mu 2$  band intensity normalised by GAPDH.  
 1078 Error bars indicate  $\pm$  SEM and significance was tested using one-way ANOVA \*\*\* $p=0.0001$ .  
 1079 (b) Blots showing levels of  $\mu 2$  protein obtained from cultured hippocampal neurons infected  
 1080 for 8-9 days with lentiviruses expressing either  $\mu 2$ -nc shRNA or  $\mu 2$ -shRNA<sub>2</sub> sequences for 8-  
 1081 9 days. GAPDH was used as loading control. Bar chart plotting the analysis of  $\mu 2$  band  
 1082 intensity normalised by GAPDH intensity. Error bars indicate  $\pm$  SEM and significance was  
 1083 tested using One-Way ANOVA, \* $p=0.019$ . (c) Representative AMPAR-mediated mEPSC  
 1084 traces from a neuron expressing  $\mu 2$ -shRNA<sub>2</sub> and an untransfected neighbour. (ci) Amplitude  
 1085 probability distributions from the neuron shown in (c). Note that reduction of AP-2 expression  
 1086 ( $\mu 2$ -shRNA<sub>2</sub>) has little effect on mEPSC amplitude. Inset, superimposed average mEPSC  
 1087 waveforms. (d) Representative AMPAR-mediated mEPSC traces from a neuron co-  
 1088 expressing Arc<sub>(WT)</sub> and nc shRNA and an untransfected neighbour. (di) Amplitude probability  
 1089 distribution from the neurons in (d). Note that co-expression of a n.c. shRNA does not  
 1090 prevent overexpression of Arc from reducing mEPSC amplitude. Inset, superimposed  
 1091 average mEPSC waveforms. (e) Representative AMPAR-mediated mEPSC traces from a  
 1092 neuron co-expressing Arc<sub>(WT)</sub> and  $\mu 2$ -shRNA<sub>2</sub> and an untransfected neighbor. (e(i))  
 1093 Amplitude probability distribution from the neurons showed in (e). Note that co-expression of  
 1094  $\mu 2$ -shRNA<sub>2</sub> prevents the effects of Arc<sub>(WT)</sub> on mEPSC amplitude. Inset, superimposed  
 1095 average mEPSC waveforms. (f) Cumulative probability distributions for cells expressing  
 1096 shRNA<sub>2</sub> (9 neurons), Arc<sub>(WT)</sub> + shRNA<sub>2</sub> (16 neurons), Arc<sub>(WT)</sub>+n.c shRNA (7 neurons), and for  
 1097 untransfected cells (12 neurons). (g) Bar chart plotting mean mEPSC amplitude for the cells  
 1098 in (f). Expression of shRNA<sub>2</sub> prevented the Arc<sub>(WT)</sub> overexpression effect of significantly  
 1099 reducing mEPSC amplitude (mean mEPSC amplitude  $15.3 \pm 1$  pA in untransfected cells,  
 1100 Arc<sub>(WT)</sub> + shRNA<sub>2</sub>  $14.3 \pm 0.8$  pA,  $p=0.52$ ). Expression of shRNA<sub>2</sub> alone had no significant  
 1101 effect on mEPSC amplitude ( $13 \pm 0.7$  pA,  $p=0.07$ ) whereas Arc<sub>(WT)</sub> + n.c. shRNA significantly  
 1102 reduced mEPSC amplitude ( $10.2 \pm 0.53$  pA,  $p=0.001$ ). (h) Bar chart plotting the mean

1103 interval between mEPSCs for the cells in (f). The error bars in (g) and (h) are SEMs  
 1104 \*\*\* $p < 0.001$ ; \*\* $p < 0.005$ . Statistical significance was tested using the Mann-Whitney test. (i)  
 1105 Amplitude probability distributions for a neuron expressing  $\mu 2$ -shRNA<sub>3</sub> and an untransfected  
 1106 neighbour and for a neuron overexpressing Arc<sub>(WT)</sub> with  $\mu 2$ -shRNA<sub>3</sub> and an untransfected  
 1107 neighbour (j). Inset, superimposed average mEPSC waveforms. (k) Bar chart of mean  
 1108 mEPSC amplitudes for untransfected cells ( $n = 8$ ), cells transfected with  $\mu 2$ -shRNA<sub>3</sub> ( $n=10$ )  
 1109 and cells transfected with Arc<sub>(WT)</sub>+  $\mu 2$ -shRNA<sub>3</sub> ( $n=6$ ). Neither expression of  $\mu 2$ -shRNA<sub>3</sub> or  
 1110 Arc<sub>(WT)</sub>+ $\mu 2$ -shRNA<sub>3</sub> significantly changed mEPSC amplitude ( $p=0.68$  and  $p=0.27$   
 1111 respectively).

1112 **Figure 7. Arc/AP-2 $\mu$  interaction is required for the Arc-mediated reduction in AMPAR**  
 1113 **mEPSC amplitude**

1114 (a) Representative AMPAR-mediated mEPSC traces from a neuron expressing Arc<sub>(WT)</sub>+ $\mu 2$ -  
 1115 shRNA<sub>2</sub>+ $\mu 2$  and an untransfected neighboring neuron. (ai) Amplitude probability distributions  
 1116 from the neurons showed in (a). Note that re-introduction of  $\mu 2$  rescued the effect of Arc<sub>(WT)</sub>  
 1117 overexpression leading to a reduction in the amplitude of mEPSC amplitudes (shift to the  
 1118 left, red trace). Inset, superimposed average mEPSC waveforms. (b) Representative  
 1119 AMPAR-mediated mEPSC traces from a neuron expressing Arc<sub>(195-199A)</sub>+ $\mu 2$ -shRNA<sub>2</sub>+ $\mu 2$  and  
 1120 an untransfected neighboring neuron. (bi) Amplitude probability distributions from the  
 1121 neurons showed in (d). Note that re-introduction of  $\mu 2$  has little effect in mEPSC amplitude  
 1122 (no shifts between black and red traces). Inset, superimposed average mEPSC waveforms.  
 1123 (c) Cumulative probability distributions for cells expressing Arc<sub>(WT)</sub> + $\mu 2$ -shRNA<sub>2</sub> + $\mu 2$  (14  
 1124 neurons), Arc<sub>(195-199A)</sub> + $\mu 2$ -shRNA<sub>2</sub> + $\mu 2$  (9 neurons) and untransfected cells (14 neurons). (d)  
 1125 Bar chart plotting mean mEPSC amplitude for the cells in (c). Expression of  $\mu 2$  rescued the  
 1126 reduction in mEPSC amplitude produced by Arc<sub>(WT)</sub> overexpression, following the  
 1127 knockdown of AP-2 by shRNA<sub>2</sub> (mean mEPSC amplitude in untransfected cells  $16.9 \pm 1.3$   
 1128 pA vs  $10.1 \pm 0.6$  pA in cells expressing Arc<sub>(WT)</sub> + $\mu 2$ -shRNA<sub>2</sub> + $\mu 2$ ,  $p=0.0001$ ). In contrast,

1129 expression of  $\mu 2$  had no significant effect on mEPSC amplitude when  $\text{Arc}_{(195-199A)}$ , which  
 1130 does not interact with AP2, was expressed together with shRNA<sub>2</sub> ( $15.9 \pm 1.7$  pA,  $p = 0.46$ ).  
 1131 (e) Bar chart plotting the mean interval between mEPSCs for the cells in (c). The error bars  
 1132 in (d) and (e) are SEMs \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ . Statistical significance was tested using the  
 1133 Mann-Whitney test.

#### 1134 **Figure 8. AP-2 is required for Arc-dependent homeostatic scaling**

1135 (a) Average mEPSC waveforms from an untransfected neuron cultured in control conditions,  
 1136 from an untransfected neuron exposed to bicuculline and from a  $\mu 2$ -shRNA<sub>2</sub> expressing  
 1137 neuron that has been incubated in bicuculline (40  $\mu\text{M}$ ; 48 hr). Note that the bicuculline-  
 1138 induced down regulation of the mEPSC amplitude was reduced in AP-2 depleted cells ( $\mu 2$ -  
 1139 shRNA<sub>2</sub> expressing cells). The untransfected neuron and the neuron expressing  $\mu 2$ -shRNA<sub>2</sub>  
 1140 that were cultured in the presence of bicuculline were neighbors in the same dish, while the  
 1141 untransfected neuron cultured in control conditions was from the same preparation. (b)  
 1142 Cumulative amplitude distribution for untransfected neurons cultured in control conditions  
 1143 (black line,  $n=10$  neurons), untransfected neurons incubated in bicuculline (red line,  $n=15$   
 1144 neurons),  $\mu 2$ -shRNA<sub>2</sub> expressing cells incubated in bicuculline (blue line,  $n=6$  neurons) and  
 1145 cells transfected with n.c. shRNA incubated in bicuculline (green line,  $n=5$  neurons). (c) Bar  
 1146 chart plotting the mean mEPSC amplitude for the cells shown in (b). Incubation in bicuculline  
 1147 significantly reduced the mean mEPSC amplitude (from  $17.3 \pm 1$  pA to  $11.9 \pm 0.2$  pA,  
 1148  $p=0.0001$ ). Expression of shRNA<sub>2</sub> significantly increased mEPSC amplitude in bicuculline  
 1149 ( $14.38 \pm 0.16$  pA,  $p=0.0001$ ) whereas n.c shRNA had significantly less effect ( $12.9 \pm 0.28$   
 1150 pA,  $p=0.007$ ). (d) Bar chart plotting the mean interval between mEPSCs for the cells in (b  
 1151 and c). The error bars in (c) and (d) are SEMs \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ . Statistical significance  
 1152 was tested using the Mann-Whitney test.

#### 1153 **Figure 9. Arc/AP-2 interaction controls synaptic strength.**

1154 The proposed model showing the mechanism by which Arc/AP-2 interaction facilitates  
1155 AMPAR endocytosis. An increase in neuronal activity promotes rapid Arc mRNA translation  
1156 and protein expression at the dendritic spines. (1) Newly expressed Arc binds to the AP-2  
1157 complex and may activate/facilitate AP-2 interaction with AMPAR at the plasma membrane.  
1158 (2) To initiate the formation of the clathrin-coated assembly AP-2 binds and recruits clathrin  
1159 to the membrane. (3-4) Arc then binds and recruits endophilin and dynamin to promote  
1160 scission of the endocytic vesicle containing the AMPAR to be targeted for either recycling or  
1161 degradation.

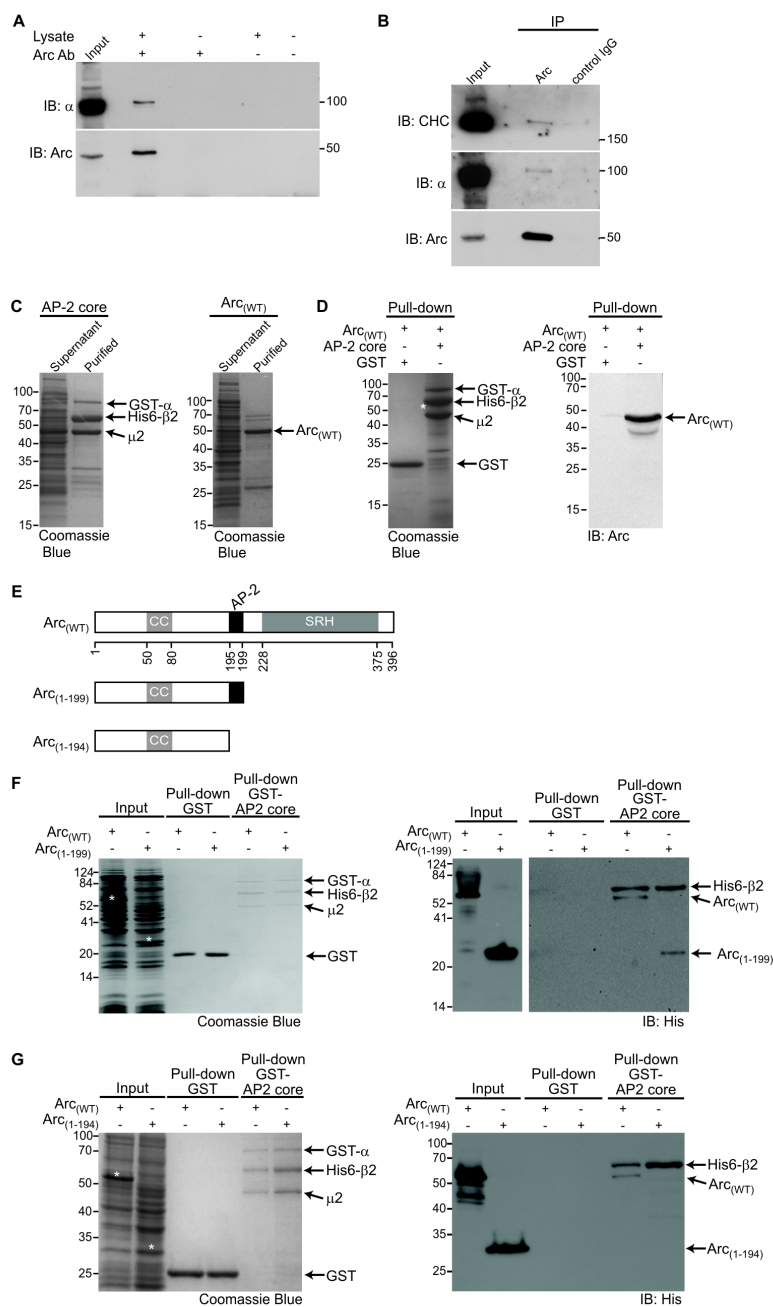


Figure 1



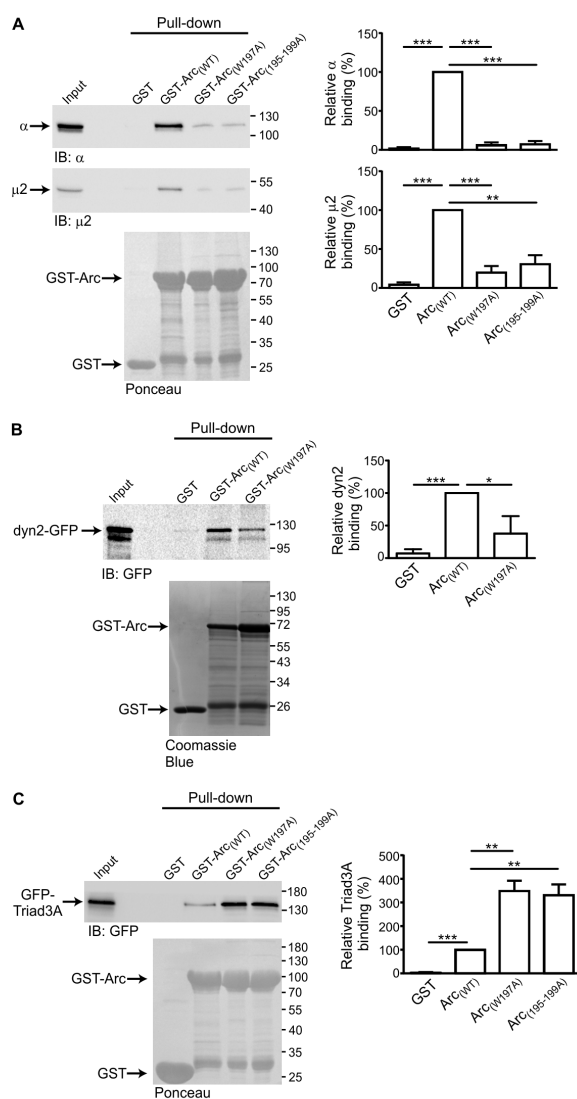


Figure 2

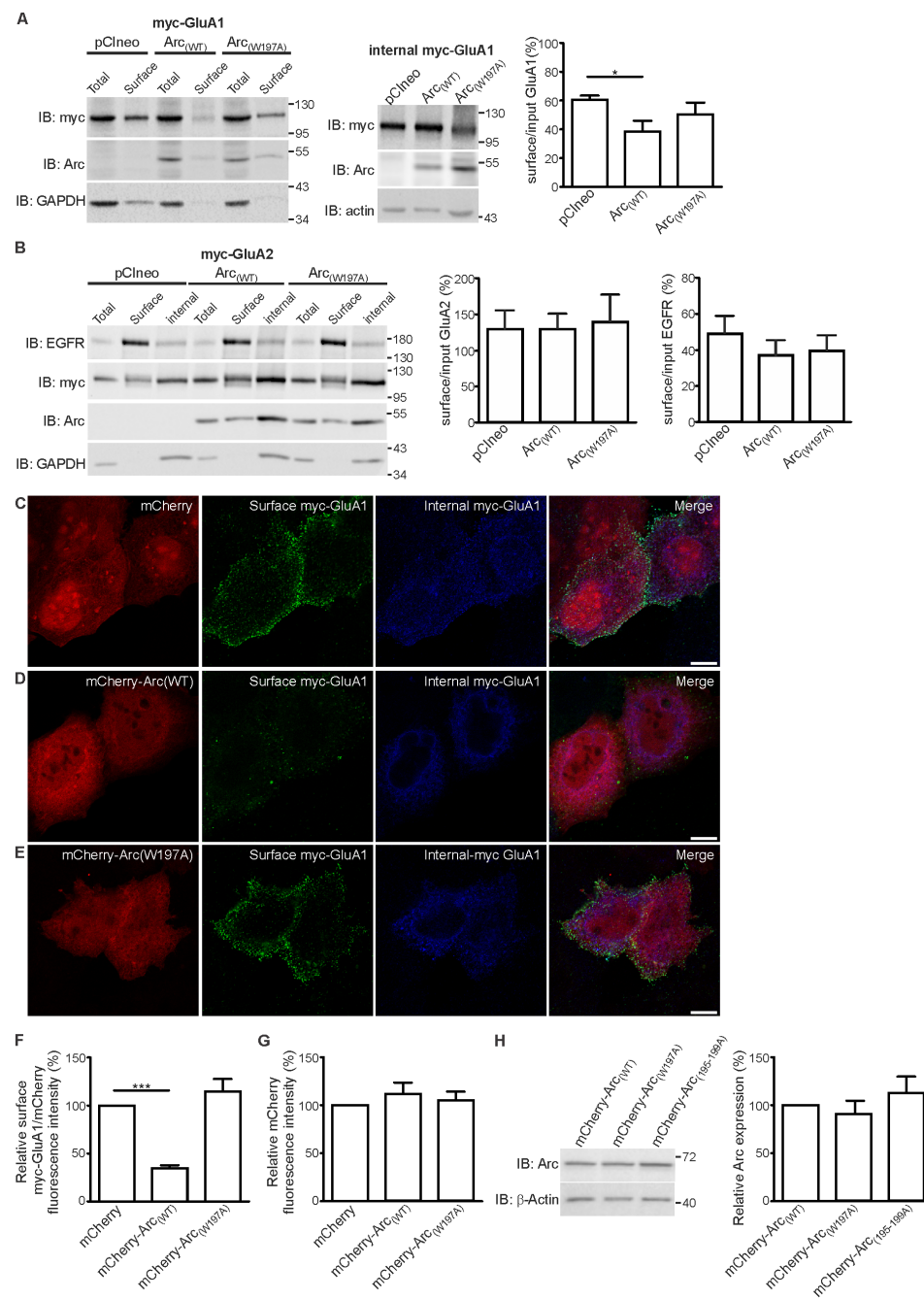


Figure 3

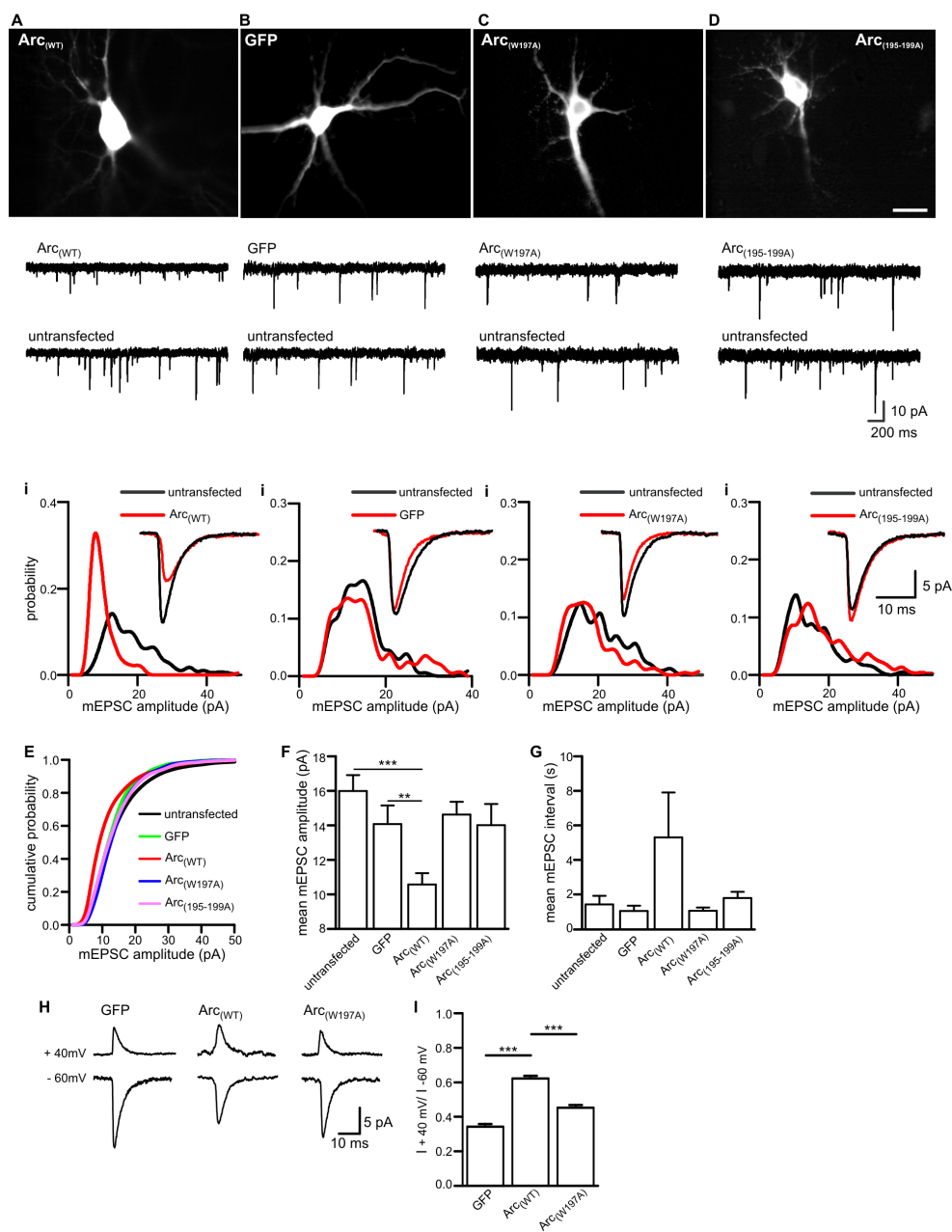


Figure 4

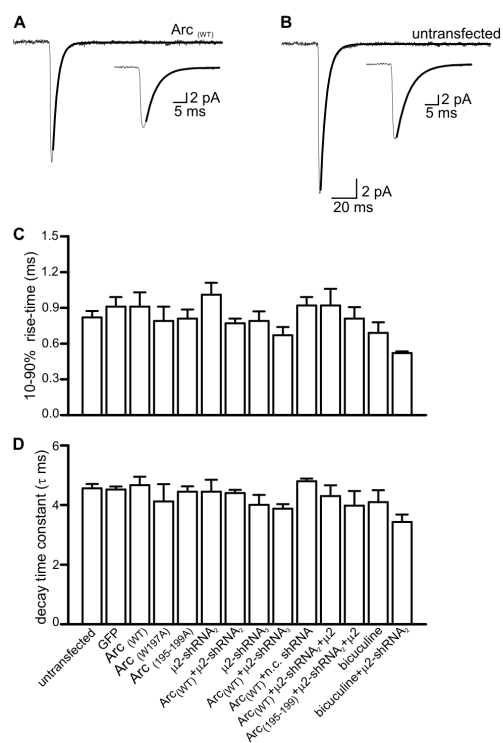


Figure 5

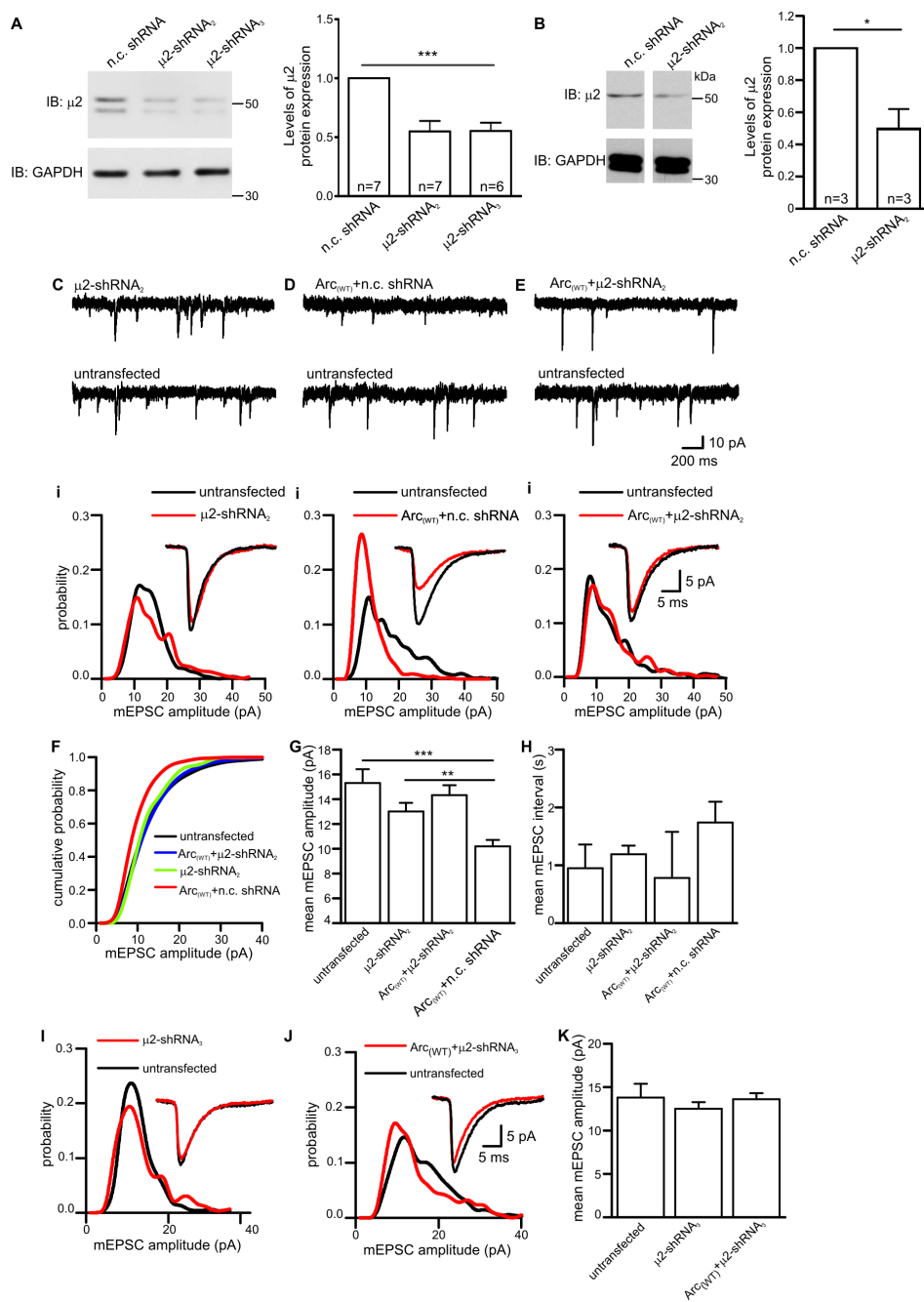


Figure 6

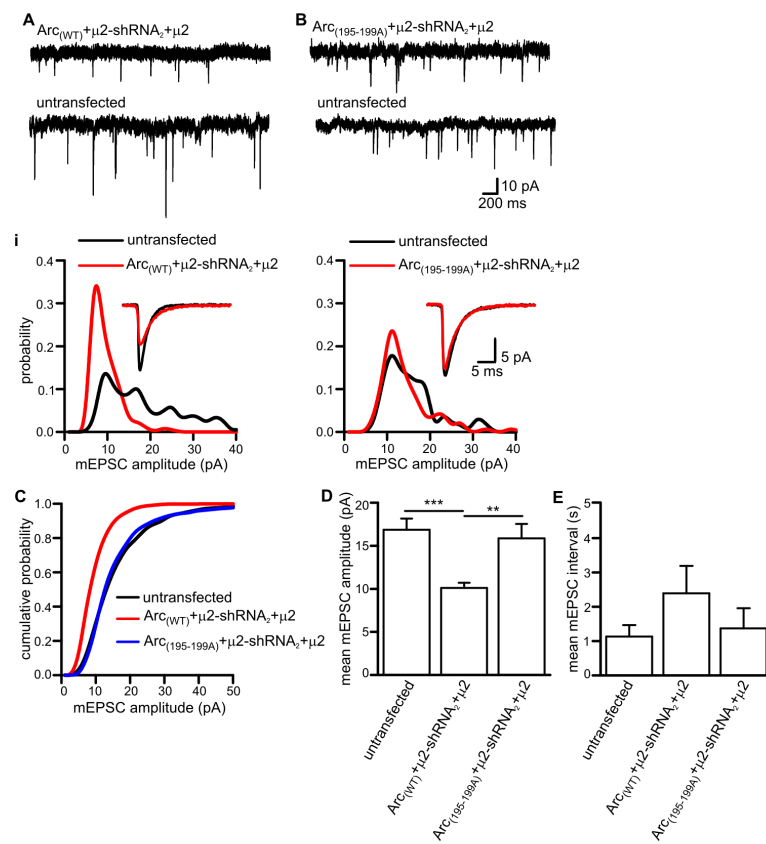


Figure 7

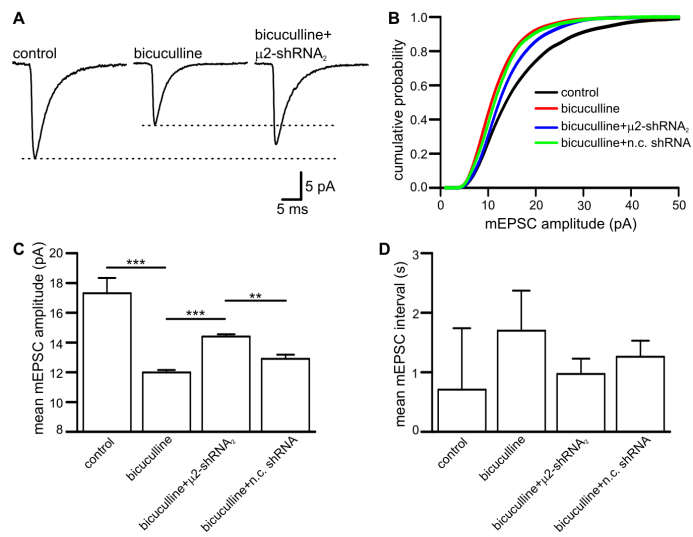


Figure 8

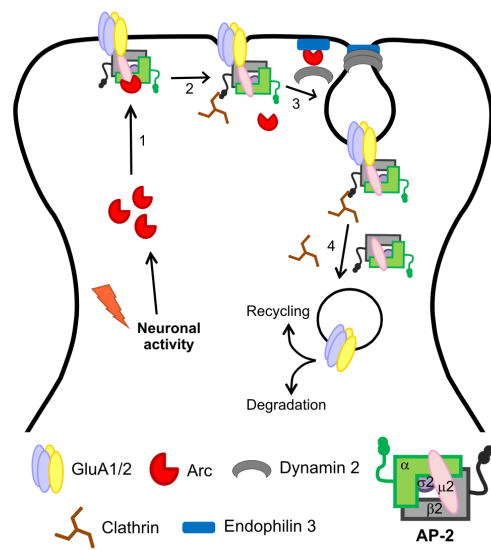


Figure 9



Table1: Statistical Analyses

Results		Data Structure	Type of Test	n numbers	Probability (P)
(Fig. 2a, top panel) IB pulldown $\alpha$	GST-Arc <sub>(WT)</sub> vs GST	Two factor, mean	t test	3/3	< 0.0001
	GST-Arc <sub>(WT)</sub> vs GST-Arc <sub>(W197A)</sub>	Two factor, mean	t test	3/3	< 0.0001
	GST-Arc <sub>(WT)</sub> vs GST-Arc <sub>(195-199A)</sub>	Two factor, mean	t test	3/3	< 0.0001
(Fig. 2a, middle panel) IB pulldown $\mu 2$	GST vs GST-Arc <sub>(WT)</sub>	Two factor, mean	t test	3/3	< 0.0001
	GST-Arc <sub>(WT)</sub> vs GST-Arc <sub>(W197A)</sub>	Two factor, mean	t test	3/3	0.0007
	GST-Arc <sub>(WT)</sub> vs GST-Arc <sub>(195-199A)</sub>	Two factor, mean	t test	3/3	0.0039
(Fig. 2b) IB pulldown dyn2-GFP	GST-Arc <sub>(WT)</sub> vs GST	Two factor, mean	t test	3/3	< 0.0001
	GST-Arc <sub>(WT)</sub> vs GST-Arc <sub>(W197A)</sub>	Two factor, mean	t test	3/3	0.0159
(Fig. 2c) IB pulldown GFP-Triad3A	GST-Arc <sub>(WT)</sub> vs GST	Two factor, mean	t test	3/3	< 0.0001
	GST-Arc <sub>(WT)</sub> vs GST-Arc <sub>(W197A)</sub>	Two factor, mean	t test	3/3	0.0055
	GST-Arc <sub>(WT)</sub> vs GST-Arc <sub>(195-199A)</sub>	Two factor, mean	t test	3/3	0.0055
(Fig. 3a) IB Surface GluA1	pCIneo vs pArc <sub>(WT)</sub>	Two factor, mean	ANOVA Tukey's	3/3	0.1284
	pCIneo vs pArc <sub>(W197A)</sub>	Two factor, mean	ANOVA Tukey's	4/4	0.5543
(Fig. 3b) IB Surface GluA2	pCIneo vs pArc <sub>(WT)</sub>	Two factor, mean	ANOVA Tukey's	4/4	>0.9999
	pCIneo vs pArc <sub>(W197A)</sub>	Two factor, mean	ANOVA Tukey's	4/4	0.9637
(Fig. 3b) IB Surface EGFR	pCIneo vs pArc <sub>(WT)</sub>	Two factor, mean	ANOVA Tukey's	4/4	0.6156
	pCIneo vs pArc <sub>(W197A)</sub>	Two factor, mean	ANOVA Tukey's	4/4	0.7621

(Fig. 3f) IF Surface GluA1	mCherry vs mCherry-Arc <sub>(WT)</sub>	Two factor, mean	ANOVA Tukey's	59/60	<0.0001
	mCherry vs mCherry-Arc <sub>(W197A)</sub>	Two factor, mean	ANOVA Tukey's	59/42	0.3438
(Fig. 3g) IF mCherry expression	mCherry vs mCherry-Arc <sub>(WT)</sub>	Two factor, mean	ANOVA Tukey's	3/3	0.5625
	mCherry vs mCherry-Arc <sub>(W197A)</sub>	Two factor, mean	ANOVA Tukey's	3/3	0.9211
(Fig. 3h) IB Arc expression	mCherry-Arc <sub>(WT)</sub> vs mCherry-Arc <sub>(W197A)</sub>	Two factor, mean	ANOVA Tukey's	3/3	0.6892
	mCherry-Arc <sub>(WT)</sub> vs mCherry-Arc <sub>(195-199A)</sub>	Two factor, mean	ANOVA Tukey's	3/3	0.4951
(Fig. 4) Arc/AP-2 interaction	Arc <sub>(WT)</sub> vs untransfected amplitude frequency	Two factor, mean	Mann Whitney	12/20	0.0002 0.47
	Arc <sub>(W197A)</sub> vs untransfected amplitude frequency	Two factor, mean	Mann Whitney	13/20	0.121 0.98
	Arc <sub>(195-199A)</sub> vs untransfected amplitude frequency	Two factor, mean	Mann Whitney	10/20	0.372 0.18
	eGFP vs untransfected amplitude frequency	Two factor, mean	Mann Whitney	7/20	0.376 0.39
(Fig. 5) cDNA constructs and mEPSC kinetics	All constructs vs untransfected rise decay	Two factor, mean	Mann Whitney	6/18	>0.05 >0.05
(Fig. 6) AP-2 requirement for Arc mediated changes in synaptic strength	$\mu$ 2-miRNA <sub>2</sub> vs untransfected amplitude frequency	Two factor, mean	Mann Whitney	9/12	0.07 0.37
	Arc <sub>(WT)</sub> + $\mu$ 2-miRNA <sub>2</sub> vs untransfected amplitude frequency	Two factor, mean	Mann Whitney	16/12	0.52 0.63
	Arc <sub>(WT)</sub> + n.c.miRNA vs untransfected amplitude frequency	Two factor, mean	Mann Whitney	7/12	0.001 0.08
	$\mu$ 2-miRNA <sub>3</sub> vs untransfected amplitude frequency	Two factor, mean	Mann Whitney	10/8	0.68 0.45
	Arc <sub>(WT)</sub> + $\mu$ 2-miRNA <sub>3</sub> vs untransfected amplitude frequency	Two factor, mean	Mann Whitney	6/8	0.27 0.14
	Arc <sub>(WT)</sub> + $\mu$ 2-miRNA <sub>2</sub> + $\mu$ 2 vs untransfected amplitude frequency	Two factor, mean	Mann Whitney	14/14	0.0001 0.37
(Fig. 7) The Arc-AP-2 $\mu$ interaction is required for Arc-mediated changes in synaptic strength	Arc <sub>(195-199A)</sub> + $\mu$ 2-miRNA <sub>2</sub> + $\mu$ 2 vs untransfected amplitude	Two factor, mean	Mann Whitney	9/14	0.46

	frequency				0.64
(Fig. 8) AP-2 is required for homeostatic scaling.	Control vs bicuculline (untransfected) amplitude frequency	Two factor, mean	Mann Whitney	10/15	0.0001 0.64
	miRNA <sub>2</sub> (bicuculline) vs untransfected (bicuculline) amplitude frequency	Two factor, mean	Mann Whitney	6/15	0.0001 0.59
	n.c.miRNA (bicuculline) vs untransfected amplitude frequency	Two factor, mean	Mann Whitney	5/15	0.007 0.29